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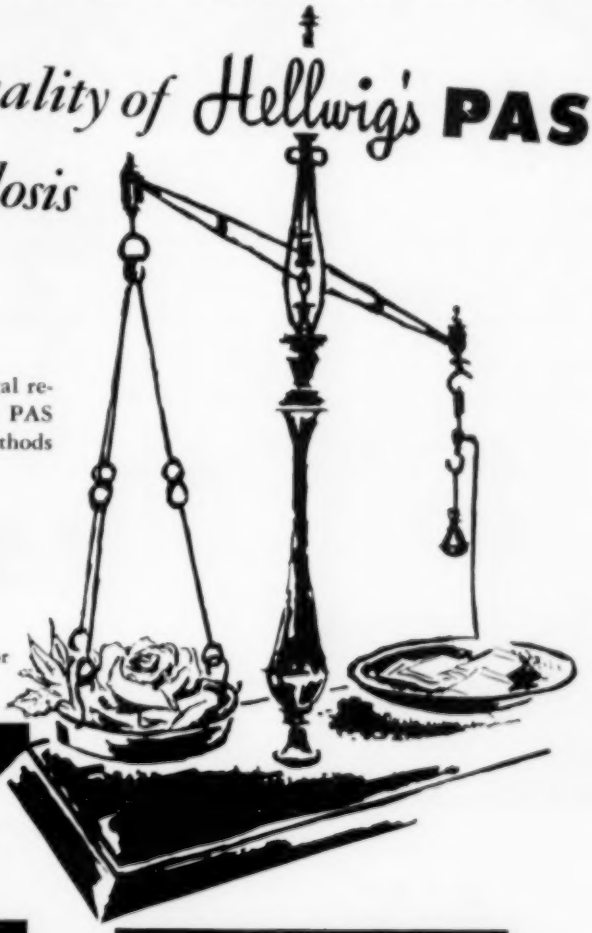

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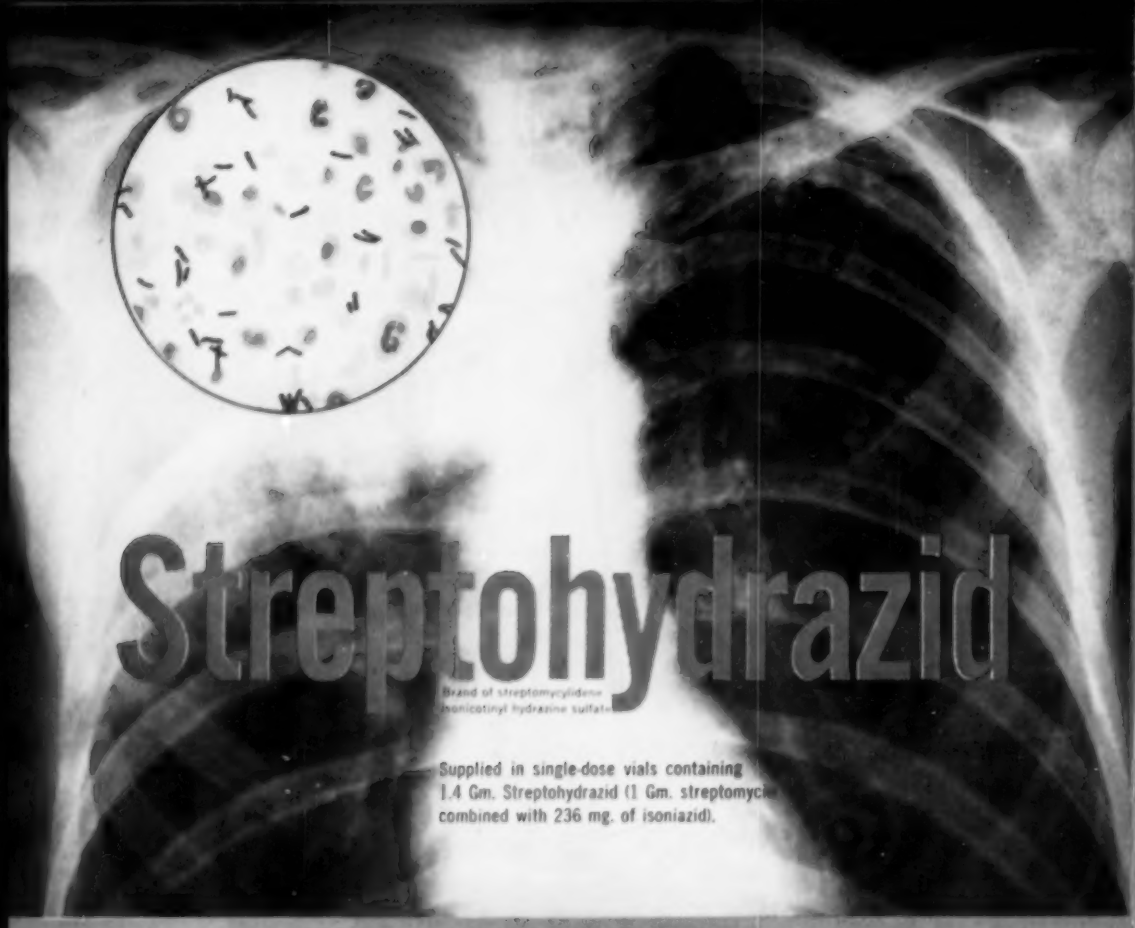

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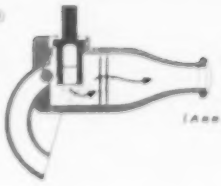

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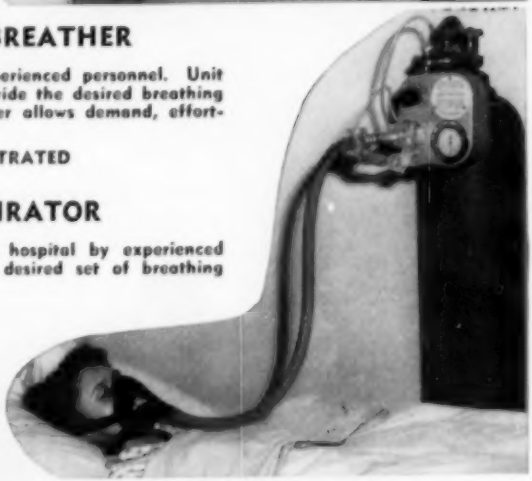

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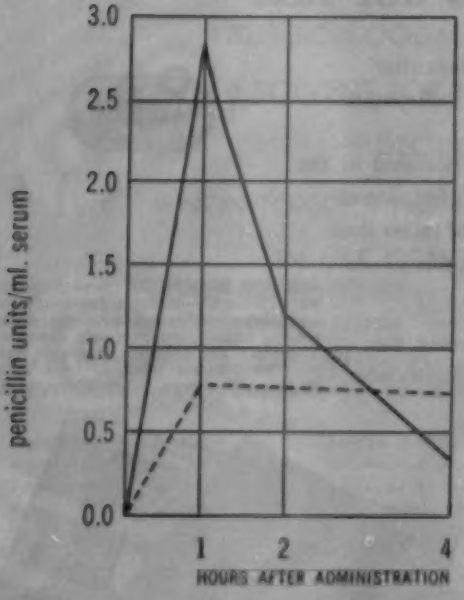

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2. Price, A.H.: Personal communication.

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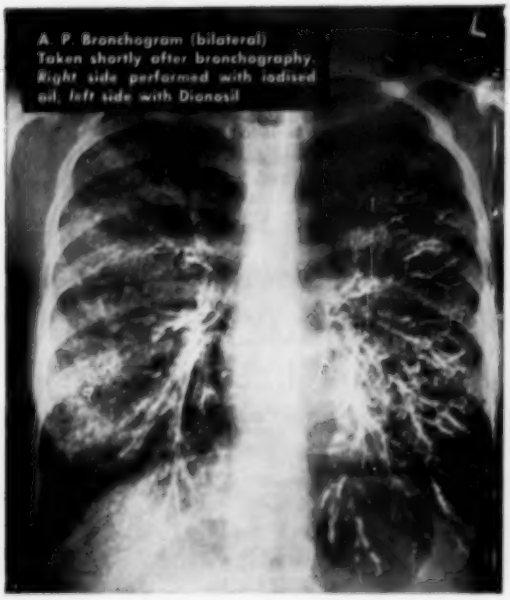

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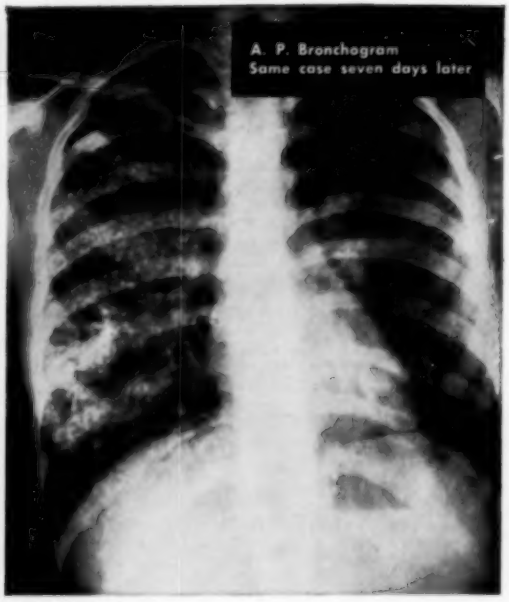

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[2] Sanders, Murray, & Soret, Manuel G. Virucidal activity of WCS-90. Antibiotics & Chemotherapy, Vol. 5, No. 11, Nov. 1955.

[3] Wolinsky, Emanuel Smith, Marjorie M. Steenken, William Tuberculocidal Activity of Clorpactin, The New Chlorine Compound. Antibiotic Medicine, Vol. 1, No. 7, July 1955.

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*Miller, J.B., et al.: Ann. Allergy, 12:611, Sept.-Oct., 1954.

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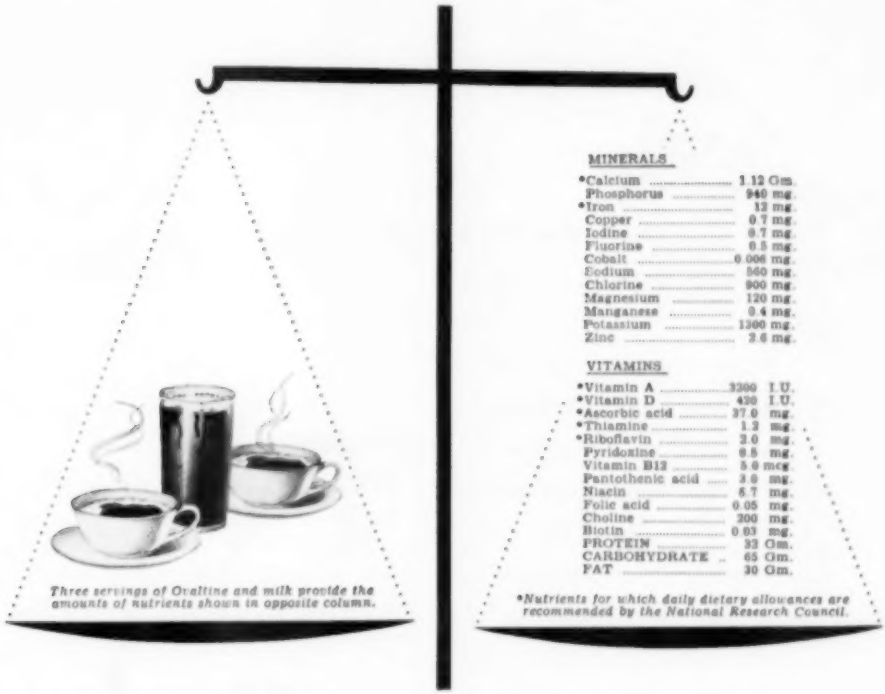

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ERRATUM

The writers of the article which appeared in the February, 1956, issue of the REVIEW (volume 73, page 229), entitled "Lymph Node Tuberculosis in Neck, Axilla, and Groin," wish to call attention to the omission of the following paragraph which should be inserted in the text at page 230 just before the paragraph beginning "The process can be arrested at this point. . . ":

> "In the next phase tubercles and caseation necrosis are present. Fibrous tissue forms about the nodes binding them together, the so-called 'periglandular fibrosis.' The swelling may be slightly smaller and the nodes are matted, firm, and not tender. The fever has disappeared."

THE AMERICAN REVIEW OF TUBERCULOSIS AND PULMONARY DISEASES

VOLUME 73 *April, 1956* NUMBER 4

THE "QUIESCENT" TUBERCULOUS BRONCHUS[1,2]

A Review with a Study of Its Surgical Connotations

KENNETH L. HARDY AND PAUL C. SAMSON

(Received for publication August 16, 1955)

INTRODUCTION

During the past two decades the progress in the treatment of pulmonary tuberculosis has effected a continuously changing pattern of thought among students of the disease. It is the purpose of this paper to re-emphasize the importance of the bronchial factor in the over-all evaluation of pulmonary tuberculosis, to stress certain hitherto unmentioned concepts relating to tuberculous tracheobronchitis, and to suggest specific recommendations for the management of the patient with this complication who is a candidate for excisional surgery.

Criteria to reduce postoperative bronchial complications will be presented on the basis of a correlative clinical, bronchoscopic, operative, and pathologic study of 105 cases in which resection was performed for pulmonary tuberculosis. The patients were all studied consecutively in the period between September, 1952, and June, 1954, on the Thoracic Surgery Service at Highland-Alameda County Hospital.

The literature on the treatment of tuberculosis during the last few years has been largely concerned with descriptions of new drugs and dosage regimens, discussions concerning the viability of the tubercle bacillus *in vivo* under prolonged chemotherapy, and reports of increasingly impressive series of surgical resections. With well-standardized techniques, pulmonary excision in tuberculosis has come to occupy a stable position in our therapeutic armamentarium and its indications are constantly being extended. Nevertheless, in spite of progressively improving mortality statistics, bronchopleural fistulas and bronchial stump ulcers continue to be reported in appreciable numbers. It is clear, therefore, that prolonged chemotherapy has not lessened the need to consider the bronchial factor, and the importance of accurate preoperative evaluation of the lower airway cannot be overemphasized.

[1] From the Thoracic Surgery Service, Highland-Alameda County Hospital, Oakland, California.

[2] Originally the theme of the 1954 California Trudeau Society Prize Essay won by the senior author. Award presented at the annual meeting of the California Tuberculosis and Health Association, San Francisco, California, April 1–3, 1954.

REVIEW OF THE LITERATURE

The pre-streptomycin era, a period of recognition and concern: Louis (1) first briefly described the pathology of tuberculous tracheobronchitis in 1836; Eppinger (2) gave a more cogent discussion; and Cornet (3) attempted a pathologic classification. Habershon (4) and Minkovsky (5) both found tuberculous involvement of the trachea in 10 per cent of routine unselected autopsies. Bugher, Littig, and Culp (6), however, are believed to be the first to have appreciated the true pathologic significance of the condition. These workers studied 122 cases of death due to pulmonary tuberculosis, and described tuberculous lesions of the trachea and major bronchi in 41 per cent of them. They concluded that, in general, the pathogenesis of the condition was on the basis of surface infection due to contact with bacillus-rich sputum. They did recognize the possibilities of peribronchial lymphatic extension and of infection by contiguous invasion of the bronchus.

In the early nineteen thirties, Eloesser (7), Haslinger (8), and Jackson and Jackson (9) each described advanced stenotic lesions of the bronchial tree in tuberculosis. In 1936, one of the present writers (10) first defined the clinical syndrome of tuberculous tracheobronchitis. The bronchoscopic appearances were classified as nonulcerative, ulcerative, hyperplastic, and fibrostenotic; the serious prognosis of the disease was accurately assessed. On the basis of this study, certain capricious and previously unexplained symptoms in tuberculous patients became more understandable. Varying degrees of bronchial occlusion produced by tuberculous tracheobronchitis were found to account for the clinical manifestations of wheezing, rhonchi, asthma-like attacks, cyanosis, and cyclic changes in temperature and sputum volume.

Likewise, cause for the various roentgenographic patterns of obstructive emphysema, atelectasis, and the rapid appearance and disappearance of "blocked" or "tension" cavities became manifest. Shipman (11) pointed out that pneumothorax was contraindicated in the presence of tuberculous tracheobronchitis because of the high incidence of atelectasis, suppuration, and empyema, and some thoracoplasty failures became readily explicable. McIndoe and associates (12) made the first systematic study of the clinical incidence of the disease by performing routine bronchoscopy in 272 patients consecutively admitted to tuberculosis hospitals. In 11 per cent of these patients bronchoscopic evidence of tuberculous involvement of the trachea and major bronchi was found.

Following the stimulus of this study, numerous articles appeared in the literature (13, 14) stressing the importance of bronchoscopy in the recognition and treatment of tuberculous tracheobronchitis; use of both local chemical and electrical cautery techniques was investigated as well as the possible value of roentgenographic therapy. In 1941, Tuttle and co-workers (15) illustrated the inadequacy of these treatments and re-emphasized the serious prognosis of tuberculous bronchitis. They again stressed the significance of the syndrome which Eloesser had described almost a decade before as "the stepchild of clinical medicine." In the study, by Tuttle and associates, one third of 92 patients with treated tuberculous tracheobronchitis were dead within one year, and the status of the tuberculosis in another third was classified as unstable. The high incidence of pneumothorax catastrophies in the presence of this entity was pointed out and the writers half-heartedly recommended thoracoplasty as a solution to the problem. Other studies (16, 17) stressed similar points. It was suggested that thoracoplasty probably should be the mainstay of therapy in these difficult cases but that a certain percentage of the patients should have pneumonectomy.

In 1944, Overholt and Wilson (18) presented the first significant series of resections in tuberculosis. Their operative mortality was 11.6 per cent in 60 cases. Thirty-two per cent of the patients were interpreted as having bronchoscopic evidence of tuberculous tracheo-

bronchitis preoperatively. The writers stated that an endobronchial lesion, regardless of its activity, was not necessarily a contraindication to excisional surgery and that the tuberculous bronchus would heal. In the light of present concepts, this is an interesting statement from the pre-streptomycin era. Meissner (19) made the first significant pathologic study of surgical specimens by examining the material from the Overholt-Wilson series and found endobronchial tuberculosis of the major bronchi in 52 per cent. Two of the patients in the group which survived operation developed bronchopleural fistula and 5 had post-resection ulceration of the bronchial stump.

The trend of thought of this era may be summarized as follows:

Tuberculous tracheobronchitis had been recognized as an entity largely through the increasing use of bronchoscopy.

Its presence explained many previous enigmas in tuberculous patients, and its serious prognosis was stressed.

Active tuberculous bronchitis was believed to contraindicate pneumothorax and, frequently, thoracoplasty; its dangers in resection were recognized in the acute phase but there was increasing agreement that, in the healed or stenotic stage, resection might be considered as an alternative to thoracoplasty.

In the ulcero-hyperplastic stages, treatment was limited to local cauterization with silver nitrate of phenol, electro-surgical desiccation, and radiation therapy; the results from these techniques were far from gratifying, as was pointed out so well by Myerson (20) and others.

The early streptomycin era, a period of complacency: Following the report on streptomycin by Waksman and his associates in 1944, its efficacy in clinical tuberculosis was demonstrated (21, 22). It was soon appreciated that the most dramatic effects of streptomycin were in miliary and meningial tuberculosis, in acute pneumonic disease, and in tracheobronchial ulceration. In these forms of tuberculosis, streptomycin demonstrated its superiority over any previous treatment. In 1947, working with both parenteral and aerosol short-term streptomycin treatment, Brewer and Bogen (23) showed complete *bronchoscopic* healing of severe ulcero-hyperplastic tuberculous tracheobronchitis in a high percentage of cases. These observations were later confirmed in a group of 62 patients (24).

Progress in antituberculous chemotherapy has been extremely rapid. The picture of tuberculous tracheobronchitis has been markedly altered since the advent of streptomycin, para-aminosalicylic acid (PAS), and isoniazid, and the development of long-term regimens of various combinations of these and other antituberculous drugs. O'Keefe (25) pointed out that, under these newer circumstances, specific tracheobronchitis now approached tuberculous laryngitis in frequency, being present in 2 to 4 per cent of *all* cases of tuberculosis. He warned, however, that two small groups of patients still continued to be severe problems: those with progressive endobronchial disease in the face of drug-resistant infections; and those with signs of bronchial obstruction and collapsed lung due to the compressive effects of extrabronchial tuberculous lymph nodes. The latter phenomenon is particularly characteristic of primary infections in children (26).

The histopathologic studies of Olson and associates (27) sharply contrasted with O'Keefe's clinical and bronchoscopic observations. Olson and his associates found that 28 per cent of specimens from minimal cases had active endobronchial tuberculosis; of the specimens from moderately and far advanced cases, 35 per cent and 48 per cent, respectively, had endobronchial lesions on microscopic study.

Coincidently with the changing scene in tuberculous tracheobronchitis under prolonged chemotherapeutic programs, an unwarranted complacency seems to have arisen regarding the bronchial factor in relation to excisional surgery.

The contemporary era, a period characterized by a renascence of concern: In 1953, Roll (28) reported a 24 per cent incidence of postoperative bronchial stump ulcer in a group of 400 resections performed between 1946 and 1951. While long-term chemotherapy was not being used during that period, it is certain that most of the patients had had some form of antimicrobial therapy. The writer related the high incidence of ulcer to the appearance of streptomycin-resistant infections and believed that under prolonged regimens the future would be brighter with respect to the complication. A more recent report from the same clinic confirms the accuracy of this prophecy (29).

Murphy and Becker (30) reported a series of 102 patients who had had short-term streptomycin protection in connection with resection procedures. The total mortality rate was 9 per cent; bronchopleural fistula occurred in 9 cases. The writers stated that pulmonary resection for tuberculosis could be performed with a reasonable mortality, but that bronchopleural fistula and late reactivation of disease were the major morbidity problems to be solved. Bronchial fistula was further emphasized by the same writers (31) when they compared the complications and mortality in 30 cases of post-resection bronchopleural fistula treated during 1945–1950 with 15 cases treated during 1951–1952. The preoperative appearances of the bronchi were not described in either paper.

In 325 consecutive resections reported by Gale and co-workers (32), there were six bronchopleural fistulas in the 18 postoperative deaths. Thirteen additional fistulas occurred among the survivors for a fistula incidence of 6 per cent. These authors, likewise, did not mention the status of the bronchus at the time of surgery.

Overholt, Wilson, and Gehring (33) reported 437 resections performed between 1934 and 1950. They compared various complications in the pre- and post-streptomycin era. Although an average decrease of more than 50 per cent in bronchial complications was noted, bronchial fistula, stump ulceration, and empyema were not eliminated in the post-drug era.

The accompanying table combines Steele's (34) statistics from the thirteenth annual Veterans Administration-Army-Navy Conference in 1954 with similar figures from the fourteenth Conference in 1955 (35). Consecutive cases are recorded below covering the period from July, 1952, to July, 1954. The reporting hospitals have maintained well-controlled chemotherapeutic protocols.

	Number of Cases		Number of Deaths		Bronchopleural Fistula			
					1954		1955	
	1954	1955	1954	1955	Number	Per Cent	Number	Per Cent
Pneumonectomy	61	58	6	8	15	25.0	8	14.0
Lobectomy	370	412	10	17	27	7.2	24	6.0
Segmental resection	433	514	2	7	21	4.8	38	7.0
Decortication with resection	125		1		8	6.4		
Totals	989	984	19	32	71	7.1	70	7.0

From analysis of this table it is obvious that bronchopleural fistula is also a continuing problem in the government hospitals. Unfortunately, there is no available information on preoperative bronchoscopic appearances in these cases.

Other figures from the report of the 1955 Conference give support to the contention of some that re-treatment cases constitute more of a fistula problem than those of patients who had had resections during the initial course of chemotherapy. Thus, in the pneumonectomy series, one-fourth had received no previous chemotherapy and one fistula developed; three-fourths of the patients were under re-treatment and seven fistulas developed. With lobectomy, slightly more than 50 per cent of the patients were receiving chemotherapy for the first time and eight fistulas developed; in the remaining 48 per cent, sixteen fistulas developed. In segmental resections, 60 per cent of the patients were receiving their initial chemotherapy and 15 developed fistulas; 40 per cent were under re-treatment and there were 23 fistulas. It is well to emphasize that the experience of the present writers is not in agreement with this general concept that previously treated patients necessarily constitute more of a problem with respect to fistulas when surgery is eventually performed.

* * * *

From the foregoing reports certain conclusions may be drawn concerning tuberculous tracheobronchitis under drug treatment.

Chemotherapy results in fairly rapid epithelial healing of the tuberculous bronchus in a large percentage of cases. This accounts for the decreased incidence of acute obstructive episodes which were seen clinically in ulcero-hyperplastic cases prior to drugs. Bronchoscopic evidence of severe ulcerative bronchial disease is seen with dramatically reduced frequency in drug-treated patients. During chemotherapy most tuberculous bronchi will, in time, assume a grossly "quiescent" appearance.

The bronchial wall itself may continue to harbor tuberculous disease in spite of mucosal healing. This is borne out by the continued finding of active microscopic mural tuberculosis in both pre- and post-drug resection specimens in an unchanged incidence. Such observations on mucosal healing tend to confirm the views of Auerbach and associates (36), and of Medlar (37) that under streptomycin treatment there may develop re-epithelization of both the broncho-cavitary junction and the entire cavity. Possibly akin to this tissue response is the "bullogenic" effect of isoniazid which has been noted by a number of observers, including the present writers, and which the French writers aptly term "chemical caseotomy" with epithelial healing.

Because of these facts, we cannot be complacent about the bronchus when selecting cases for excisional surgery. Strict attention to the bronchial factor, it is believed, will materially reduce the incidence of bronchopleural fistula post-operatively. Until this point is appreciated, the incidence of fistula will remain inordinately high.

THE "QUIESCENT" TUBERCULOUS BRONCHUS

What then, in terms of practical criteria, must we apply to the bronchus when considering resection, remembering that the decision is made strictly on bronchoscopic grounds? In concept, the quiescent bronchus is one in which the desired state of "inactivity" or "healing" has been attained. It is an infected bronchus which is safe to transect. Bronchoscopically, such a bronchus will show an intact mucous membrane which is usually pale, but may be thickened,

opaque, and thrown into longitudinal folds. Septal blunting is frequent. Fibrostenosis may or may not be present. By contrast, weeks or months before, this same bronchus would have shown any or all of the accepted signs of active specific infection, such as, visible ulceration; granulation tissue; congested, oozing, granular mucosa (frequently defined as "red hot" and "beefy"); extensive edema; or ulcerostenosis.

Is it reasonable to insist that the bronchus be quiescent prior to resection? An analysis of the surgical results at the Highland-Alameda County Hospital suggests that such a policy is wise. The consecutively operated cases which had accumulated during a twenty-two-month period now have been followed for more than a year after resection. Similar criteria used in the determination of suitability for resection, both in private practice and in two other service hospitals during the same period, have strengthened the writers' belief in this concept.

The techniques of bronchial closure and coverage used by the present writers are evidently similar to those of most thoracic surgeons. Briefly stated, the procedures consist of ensuring a "flush" bronchial amputation to obviate stasis of secretions and pocketing; the use of meticulous on-end interrupted fine silk sutures; and a general practice of reinforcing the closure with areolar tissue or pleura unless it seems likely that the suture line will naturally be covered by adjacent living pulmonary or mediastinal tissue.

OBSERVATIONS

One hundred and five patients underwent resection from September, 1952, through June, 1954. There were 10 pneumonectomies, 55[1] lobectomies, 33 segmental resections, and 7 subsegmental resections.

Thirty-seven patients were classified as poor risk by the anesthesiology service; 19 were considered "pure salvage attempts" by the referring physicians. Of the latter, all but 3 are now noninfectious. Forty-seven patients (45 per cent) were on re-treatment regimens of chemotherapy. Contrary to Murphy's opinion, it is not believed that the re-treatment status alters the percentage of surgical bronchial healing, *provided* quiescence has been reached. Six patients of the entire series showed a quiescent bronchus but their strains of tubercle bacilli were resistant to isoniazid, streptomycin, and PAS when they were being considered for resection. These patients were successfully protected by pyrazinamide during the surgical period.

There were no stump breakdowns (bronchopleural fistulas) and no stump ulcers in the entire series. The single death occurred five days after extrapleural pneumonectomy of a patient who had had a bronchopleural fistula and empyema following lobectomy across a "red" bronchus in 1950.

At the time of operation all of the 105 patients met the bronchoscopic criteria for a normal or a quiescent, i.e., transectable, bronchus. Microscopic examination of the surgical specimens showed that 52 (47.5 per cent) had recognizable

[1] Includes 16 patients with segmentectomy and one patient with bronchial anastomosis in addition to lobectomy.

tuberculous tracheobronchitis in the proximal resected bronchus. Of the 38 considered on bronchoscopic examination to show specific bronchial disease, there was eventual microscopic correlation in 33 (87 per cent) (figures 1 and 2). Five showed only nonspecific inflammation microscopically (figure 3). Thus, the bronchoscopic diagnosis of tuberculous infection was correct nearly nine times out of ten.

Conversely, in 67 of the 105 patients the bronchus was considered normal throughout the course of treatment, yet 19 (28.4 per cent) were proved to have specific bronchial disease on microscopic examination of the resected specimen (figure 4). This may be classified as a bronchoscopic "error" in estimating normalcy, once in four times. Practically, however, all of these bronchi had an intact mucosa and healing was therefore certain, especially under the continuing protection of drugs. The good healing potential of this type of bronchus is further borne out in 5 cases in the series which were explored as carcinoma suspects without drug protection. All patients healed, yet 2 showed active submucosal tuberculous bronchitis on microscopic examination.

Of the 38 patients whose diagnoses were active tuberculous bronchitis at some time prior to resection, only 9 (23.6 per cent) achieved quiescence in a minimum of six months on the usual regimen of bed rest and streptomycin-PAS. Twenty-nine (76.3 per cent) required additional chemotherapy as follows:

> Isoniazid therapy was added in all 29 patients and 20 achieved a quiescent bronchus within three months. In 7 patients, pre-resection thoracoplasty was required as a further aid for bronchial healing (figures 5–8). In 2 patients, aerosol therapy and repeated bronchoscopies were successful in establishing improved cavitary drainage and concomitant bronchial quiescence.

As stressed above, adherence to the concept of a quiescent bronchus likewise has been valuable on the writers' two other hospital services. On the Thoracic Surgery Service in San Joaquin General Hospital at Stockton, California, where the tuberculosis surgery is performed for Bret Harte Sanatorium, 28 resections were performed from 1946 through 1951 (38). There were four bronchial fistulas. From January, 1952, through May, 1955, 46 additional resections were performed and there were no fistulas. Ninety-five consecutive resections were performed at the Veterans Administration Hospital, Livermore, from 1946 through 1951 (39). Six patients developed bronchial fistulas. From July, 1952, to January, 1955, an additional 100 pulmonary resections for tuberculosis were performed. There was one postoperative empyema without demonstrable fistula.

If the pale or quiescent bronchus is safe to transect even though endomural disease exists, is the incidence of bronchial fistula increased if the red or active bronchus is transected? At Highland Hospital during the twenty-six-month period, June, 1950, through August, 1952, 101 resections were performed in tuberculous patients and there were eight bronchopleural fistulas (7.8 per cent). In 18 cases, the immediate pre-resection bronchoscopic note stressed a "red" or "beefy" bronchus. It is significant that all of the eight instances of bronchopleural fistula fell within this group. Only one was a re-treatment case. Thus 44

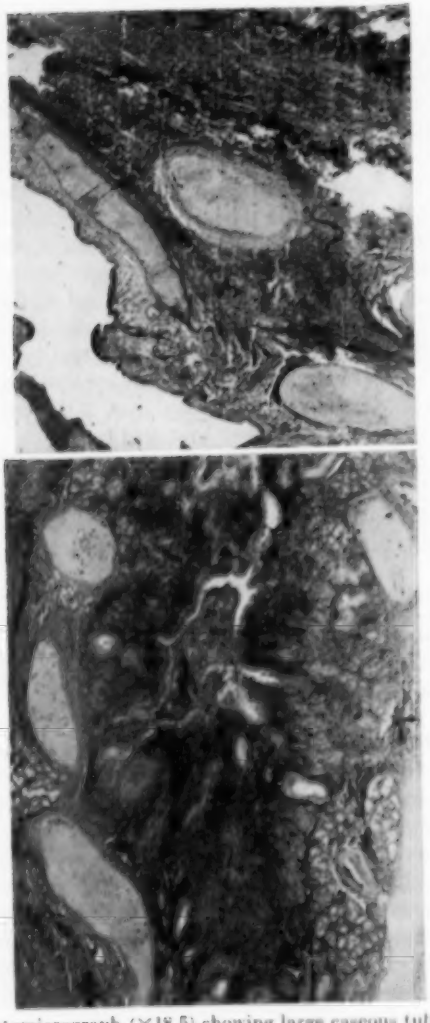

FIG. 1 (Top). Photomicrograph (×18.5) showing large caseous tubercle in adventitia of transected bronchus. Epithelium is intact. Patient previously had bronchoscopic diagnosis of acute tuberculous tracheobronchitis with ulceration. On antimicrobial therapy the bronchus became "quiescent" in seven weeks; was transected without incident. Note that mucosa had healed but mural tuberculosis persists.

FIG. 2 (Bottom). Photomicrograph (×23) showing extensive submucosal infiltration of lymphocytic tissue studded with tubercles, with resultant bronchostenosis of transected apical segment bronchus. The diagnosis had been chronic tracheobronchitis; bronchus considered "quiescent"; healed *per primum* following apico-posterior resection of right upper lobe.

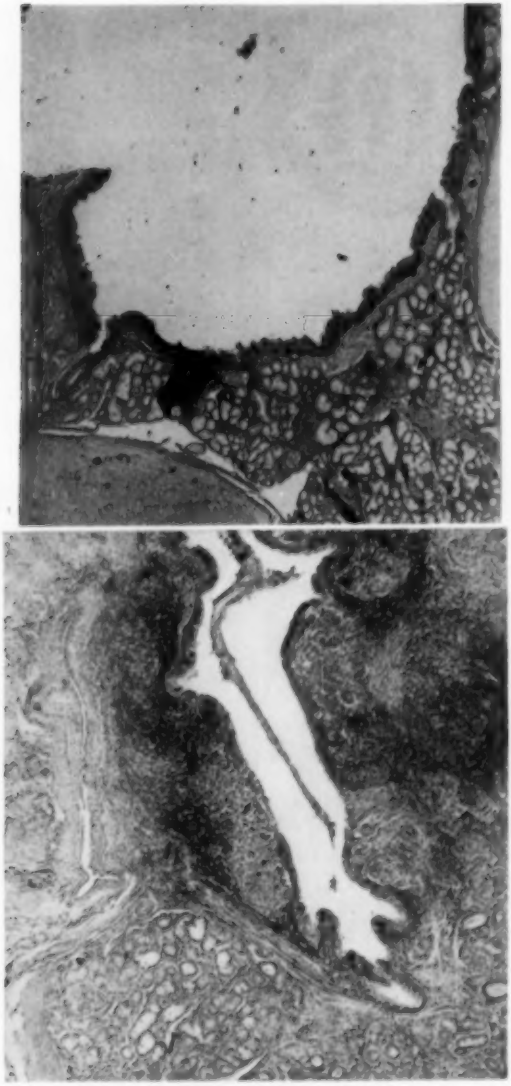

FIG. 3 (Top). Photomicrograph (×23). This is an example of chronic nonspecific bronchitis diagnosed as tuberculous tracheobronchitis bronchoscopically. Patient had extremely red and edematous right upper lobe bronchus with clinical evidence of tuberculous tracheobronchitis. In spite of streptomycin-PAS, improvement was slow during three months. After addition of isoniazid to regimen, the bronchus became bronchoscopically "quiescent" in six weeks. Note only diffuse lymphocytic infiltration in mucosa and submucosa of transected bronchus. Bronchus healed *per primum* after transection.

FIG. 4 (Bottom). Photomicrograph (×35.5). Note submucosal tubercles and giant cells in wall. Mucosa shows squamous metaplasia. Bronchus was thought normal from outset. It healed *per primum*. The patient had been on long-term chemotherapy.

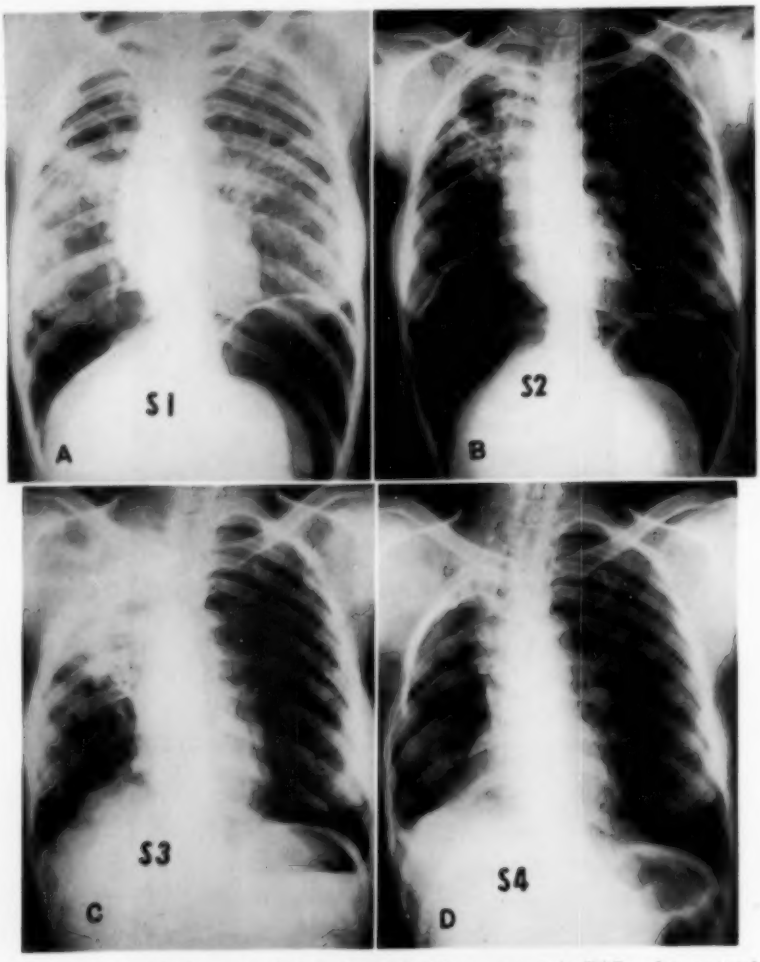

FIG. 5 *A*. Patient had previously been treated with streptomycin-PAS under sanatorium management for right upper lobe cavitary disease which responded with cavity closure and reversal of infectiousness. The patient left the hospital against medical advice and discontinued treatment. One year later she returned and presented the condition illustrated. The patient was toxic and moribund at this time.

B. After one year of retreatment with streptomycin-PAS-isoniazid, the patient was dramatically improved, but sputum continued infectious, and a wheeze and giant right upper lobe cavity persisted. Bronchoscopy revealed continuing redness and ulceration of the right upper lobe bronchus. This improved somewhat on aerosol therapy, but not to the point of quiescence.

C. Within four weeks after a three-rib thoracoplasty and discontinuance of pneumoperitoneum, the bronchus became quiescent; cough and wheeze were markedly improved.

D. Resection of the right upper lobe and lateral segment of the right middle lobe was carried out five weeks after thoracoplasty, and six weeks after the bronchus was grossly "active," without complications. The patient's disease is noninfectious eighteen months later. She is still receiving daily isoniazid-PAS. The bronchus in this case demonstrated endomural tuberculosis on microscopic examination.

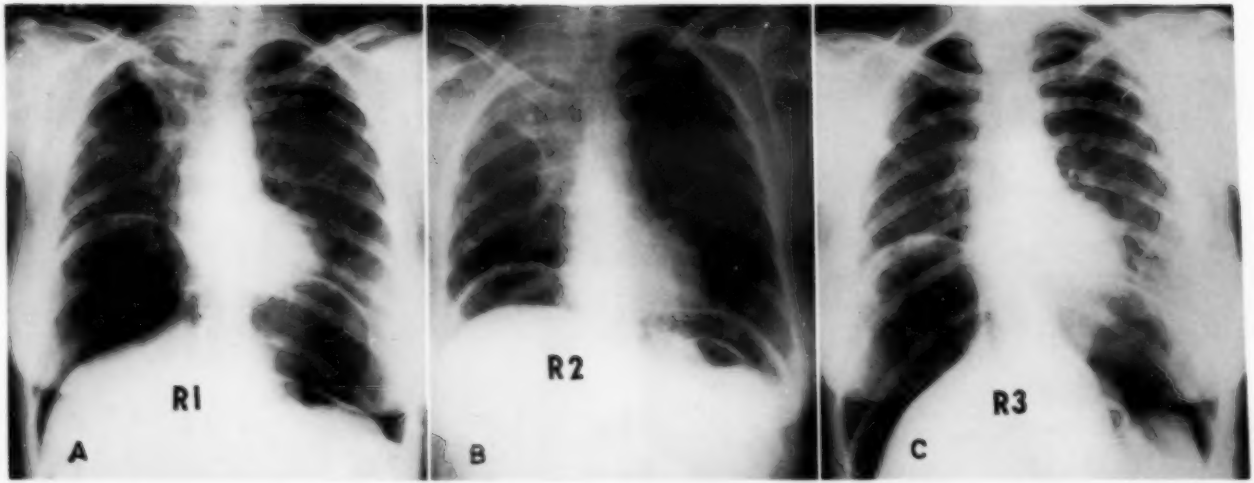

FIG. 6 *A*. Contracted cavitary right upper lobe; sputum infectious. In spite of long-term (fourteen months) streptomycin-PAS, and isoniazid for an additional five months, the bronchus continued to be red and edematous. Right upper lobectomy indicated, but bronchus not quiescent.

B. Following a two-rib thoracoplasty sputum volume and wheeze were markedly reduced. Two weeks later bronchus was bronchoscopically quiescent and was, therefore, considered transectable.

C. Three weeks following thoracoplasty and only four weeks after a beefy ulcerated bronchus was known to be present, right upper lobectomy was performed without incident. The course was uneventful; the patient was stable and sputum was noninfectious fifteen months post resection.

FIG. 6 *D*. Photomicrograph (×18.5). Note extensive tuberculous endobronchitis with regenerating mucosa.

per cent of the bronchi described as red, granular, beefy, or ulcerated immediately *prior* to resection developed major postoperative fistulas (figures 9–11).

All of the 18 patients had been on multiple-drug therapy prior to resection for a minimum of five months; 3 were on re-treatment regimens. Sixteen of the eighteen resected specimens revealed specific tuberculous tracheobronchitis on microscopic examination; the other two showed nonspecific inflammatory change.

It is obvious that in many of the patients treated surgically prior to September, 1952, transection took place, when a quiescent bronchus, as the term is now applied, had not been achieved. When bronchi with red or granular mucosa are transected, a few will heal without drug therapy and a few more will heal with drugs, but it is within this group that fistulas will develop. If, however, one adheres to the principle of achieving quiescence within this group, a marked reduction in postoperative bronchial complications will result. Approximately 10 per cent of red bronchi as viewed bronchoscopically will show only nonspecific bronchitis on pathologic examination. These changes are undoubtedly due to the trauma of harassing cough, the mechanical effect of prolonged drainage of sputum over the mucosa, or possibly to torsion factors. While a certain prediction cannot be made on bronchoscopic examination alone, nevertheless, to be safe, one is obliged to consider all red bronchi as tuberculous in the tuberculous patient. In the opinion of the writers, biopsy is neither necessary nor justified in attempting to make this differentiation.

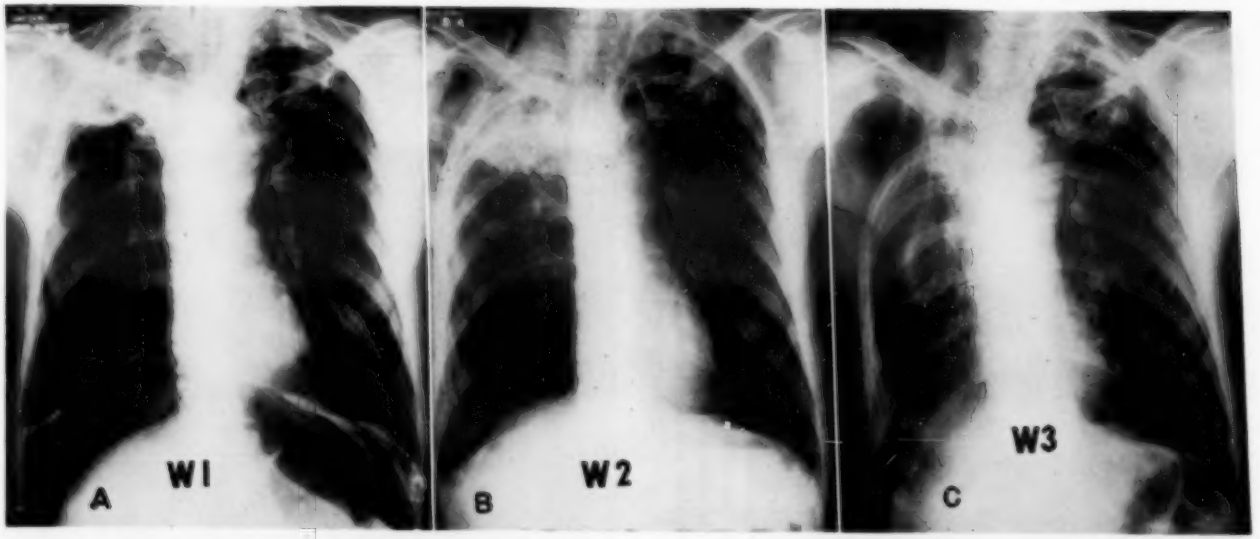

FIG. 7 *A*. Chest roentgenogram of a 61-year-old janitor who had been on pneumoperitoneum for five years and on streptomycin-PAS therapy for twelve months, with added isoniazid for three months, without cavity closure. Note marked tracheal deviation. Bronchus was "red hot" despite above therapy. Because of age and emphysema, resection of the destroyed, functionless upper lobe was the plan, rather than classical thoracoplasty which, it was believed, would sacrifice too much functionally valuable lung.

B. The bronchus was prepared for the definitive resection by a preliminary two and one-half-rib thoracoplasty. Following this, the right upper lobe bronchus became quiescent, and tracheal deviation was improved.

C. Three weeks following thoracoplasty, resection of the destroyed right upper lobe was carried out through the bed of the resected fifth rib. Because of fear of overdistention of the remaining lung tissue in this emphysematous man, more of the third and fourth ribs were removed concomitantly to effect a "tailored" five-rib thoracoplasty. The patient's convalescence was uneventful and he has been working at his original job, with sputum noninfectious, for the past seven months. His bronchus demonstrated tuberculous tracheobronchitis of the wall on microscopic examination although the mucosa was intact.

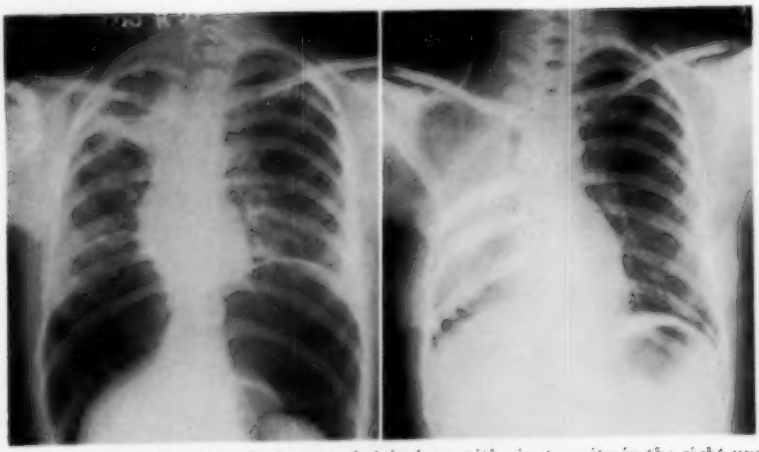

FIG. 8 *A*. Multiple cavities in destroyed right lung with giant cavity in the right upper lobe. The left lung was always stable. The bronchus was ulcerated and "beefy" red in spite of six months' drug therapy. Pneumonectomy was indicated, but bronchus was too "hot" for resection.

B. Bronchus became quiescent after a two-stage six-rib thoracoplasty, following which pneumonectomy was performed eight weeks later without incident. The course has been uncomplicated since.

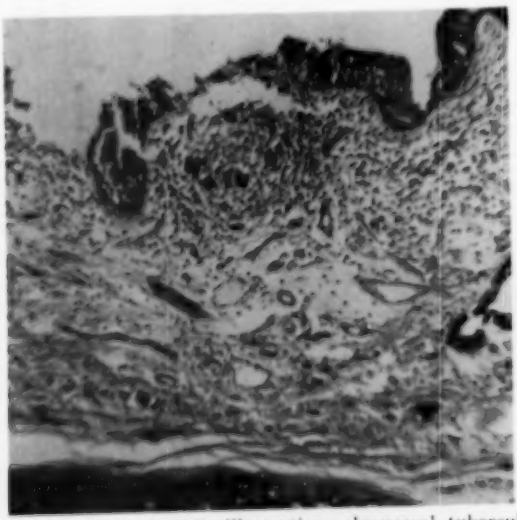

FIG. 8 *C*. Photomicrograph (×35.5), illustrating submucosal tuberculosis in proximal bronchus of resected specimen.

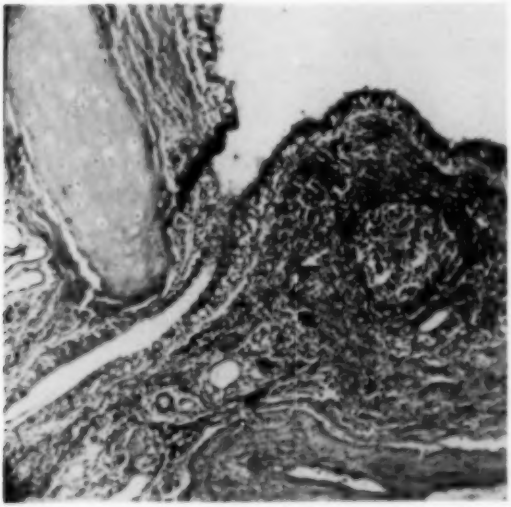

FIG. 9. Photomicrograph (×23) of a patient with a red, ulcerated bronchus at the teim of resection in 1951 who developed an immediate postoperative fistula. Note the similarity of the endomural microscopic disease to those cases of bronchoscopically quiescent bronchi, except that under the microscope there is uniformly more cellularity and patchy mucosal ulceration.

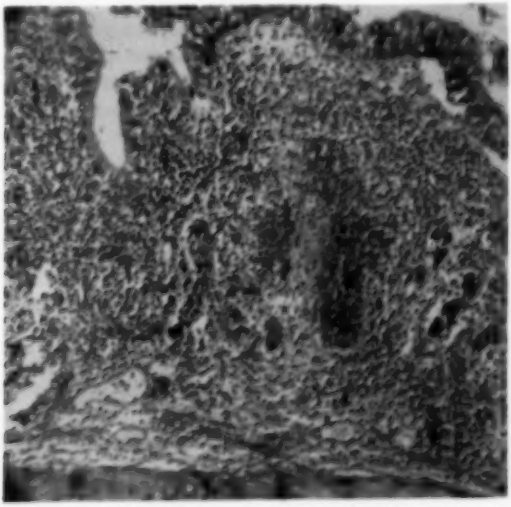

FIG. 10. Transection took place across a red or "active" bronchus in 1951 with early stump breakdown and fistula formation. Note ulcero-hyperplastic mucosa and caseating submucosa.

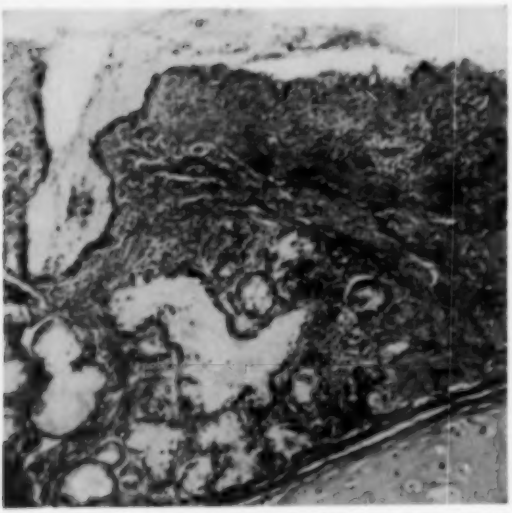

FIG. 11. The immediate pre-resection bronchoscopic note stressed a beefy red, ulcerated mucosa. Lobectomy was followed by early bronchopleural fistula. Note that the proximal resected bronchus shows a sloughing, caseating mucosa and submucosa. This condition is another example of why red bronchi are dangerous to transect in the tuberculous patient.

MEANS OF ACHIEVING THE QUIESCENT BRONCHUS

Prolonged chemotherapy is the best method for achieving the quiescent bronchus. In general, streptomycin-PAS is employed as long as the pulmonary lesion continues to clear. The maximum benefit to the bronchial lesion to be obtained by chemotherapy is usually evident within a six- to twelve-month period. If the pulmonary lesion is then considered for resection but the bronchus is still inflamed, isoniazid is added in an average dosage of 300 mg. per day. Recent evidence suggests that isoniazid doses as high as 800 mg. per day may be even more beneficial. In the experience of the writers, no more than three months on added isoniazid therapy for the bronchial lesion is justified in view of the importance of operating on the pulmonary disease without too great delay.

Further steps which may be employed to achieve a quiescent bronchus are: extrapleural thoracoplasty; the abandonment of unsuccessful pneumothorax and pneumoperitoneum; Monaldi catheter drainage and cavernostomy either alone or as a supplement to thoracoplasty; and aerosol nebulization techniques, both continuous and intermittent. The last-named procedures are most frequently used in conjunction with repeated bronchoscopies and an intensive course with broad spectrum antimicrobials. The slightly differing indications for employing these procedures may overlap. Nevertheless, their use singly or in combination decreases the amount of sputum; decreases the size of giant cavities and does away with tension characteristics; corrects impaired bronchopulmonary drainage and obviates the possibility of secondary pyogenic infection within the lung.

The most frequently used of these ancillary measures is thoracoplasty, preferably performed while the patient's infection is still drug-susceptible. This is a tailoring procedure and not necessarily a classical thoracoplasty, although staged rib removals up to six ribs may occasionally be advisable. Often giant upper lobe cavities are partially closed and excessive amounts of sputum are greatly reduced. In all of the 7 cases in which this scheme was followed, the bronchus became quiescent and early pulmonary excision was carried out. Two of the 7 cases met the bronchoscopic criteria following a three-rib thoracoplasty, and lobectomy was performed across a quiescent bronchus within four weeks of the time when acute tracheobronchitis was last seen bronchoscopically. In both patients operative wounds healed *per primum*. It appears, therefore, that, when resection is otherwise indicated, it should be performed as soon as possible after a quiescent bronchus has been achieved. This usually allows for effective drug protection to continue for a prolonged period following operation.

Both Monaldi drainage and cavernostomy have been used on rare occasions in lower lobe cavitary disease when extensive thoracoplasty would functionally sacrifice normal lung. These procedures are effective in reducing the amount of infectious sputum pouring over the inflamed bronchus to a degree at which quiescence will occur and resection is then safe. If a patient's disease is unsuitable even for thoracoplasty, external drainage of an upper lobe tension cavity will also cause bronchial improvement.

Occasionally the adjustment or abandonment of reversible collapse procedures will reduce bronchial tree distortion sufficiently to permit adequate mechanical drainage of parenchymal foci and blocked cavities.

Continuous aerosol techniques, using the Mistogen apparatus (40) containing a surface wetting agent (Triton WR® 1339) and a bronchodilator (Isuprel®), have been employed in conjunction with repeated bronchoscopies and tetracycline and related compounds. In difficult lower lobe or thoracoplasty-failure cases, in which active endobronchial disease has been unresponsive to chemotherapy, these maneuvers have shown promise in speeding bronchial repair by improving parenchymal drainage and reducing secondary infection.

Mention should be made of steroid hormones only to condemn them. Their use in the attempt to salvage two patients with severe tuberculous bronchitis at the same institution during 1950 and 1951 was reported by Meyer, Kinsell, and Parsons (41). Both patients died postoperatively, one from bronchopleural fistula and the other from acute cardiac failure. Each surgical specimen showed extensive caseating endobronchitis without attempt at healing in spite of the achievement of a visibly quiescent bronchus preoperatively. In this instance, the concept of the quiescent bronchus was false, presumably because the anti-inflammatory effect of the hormones masked the relentless nature of the advancing tuberculous process.

* * * *

It is apparent that the primary healing of tuberculous bronchi may be uniformly attained by strict adherence to certain principles. Foremost of these is the absolute demand for a quiescent bronchus before excision is undertaken. Bronchi that appear normal in a tuberculous patient often show endobronchitis

on microscopic examination (25 per cent in the present series), yet are no problem in healing. Likewise, the bronchus of a healed fibrostenosis as shown by Gebauer (42), Paulson (43), and others is no problem. It has now been learned that the quiescent bronchus is also no problem. Problems arise when the inflamed bronchus in a tuberculous patient is ignored. Although such a reddened and inflamed bronchus may well be due to chronic nonspecific irritation, it should be treated as energetically as the parenchymal focus itself, if resection is to be undertaken and distressing morbidity avoided.

SUMMARY

The history of the thinking on tuberculous tracheobronchitis has been reviewed and three periods are recognized: (*a*) the pre-streptomycin era, a period of recognition and concern; (*b*) the early streptomycin era, a period of complacency; and (*c*) the contemporary era, a period characterized by a renascence of concern.

The tendency is noted of antituberculous chemotherapy to cause epithelial surface healing of the bronchus while submucosal and mural lesions persist.

Excluding gross technical difficulties, the status of the tuberculous bronchus at the time of transection is the chief factor which determines whether healing will take place. For all practical purposes, the condition of the bronchus can be accurately determined by a competent bronchoscopist. The concept of the "quiescent" bronchus is presented. Although not a "healed" bronchus in the histopathologic sense, it will heal after transection in the surgical sense. Its bronchoscopic appearance is presented and various therapeutic techniques to achieve it are described.

It has been shown that bronchi with active tuberculous disease of the mucosa are the ones in which surgical relapse with fistula formation tends to occur after transection. Such bronchi have been variously described as ulcerated or "red hot" and "beefy."

It is suggested that the "quiescent" bronchus be used as an additional criterion in determining the indications for pulmonary resection along with the status of cavities and sputum and the roentgenographic findings. Attainment of the quiescent state of the bronchial disease is the most important single factor in reducing bronchopleural fistula and bronchial stump ulcer following resection procedures for pulmonary tuberculosis.

The wisdom of this practice is demonstrated in the achievement of 105 consecutive cases of pulmonary resection without a bronchial stump complication which have now been followed for more than one year.

SUMARIO

El Bronquio Tuberculoso "Quiescente": Estudio de Sus Connotaciones Quirúrgicas

Repasada la historia de los conceptos de la traqueobronquitis tuberculosa, reconócense tres períodos: (*a*) era pre-estreptomicina—reconocimiento y preocupación; (*b*) era temprana de la estreptomicina—¿complacencia?; (*c*) era contemporánea—renacimiento de la preocupación.

Nótase la tendencia de la quimioterapia antituberculosa a producir curación superficial del epitelio del bronquio sin mayor alteración en las lesiones submucosas y parietales.

Dejando aparte las grandes dificultades técnicas, el estado del bronquio tuberculoso al hacer el corte transversal constituye el principal factor con respecto a si sobrevendrá o no la cicatrización. Para todos los fines prácticos, el estado del bronquio tuberculoso puede determinarlo con exactitud un broncoscopista competente. Preséntase el concepto del "bronquio quiescente", que, aunque no es un bronquio "cicatrizado" en el sentido tuberculoso, sí cicatrizará después del corte transversal en el sentido quirúrgico. Describese su aspecto broncoscópico y menciónanse varias técnicas terapéuticas para lograr tal resultado.

Demuéstrase que los bronquios con afeccion tuberculosa activa de la mucosa son aquéllos en que suele aparecer, después de la transección, recidiva *quirárgica*, con formación de fístula. A esos bronquios se han aplicado varios términos, como ulcerados, "rojo vivos" y "carnosos."

Sugiérese que, para determinar el "punto focal" en la resección pulmonar se utilice el bronquio "quiescente" como *cuarta* pauta, junto con el cierre de las cavernas, el viraje del esputo y la estabilidad radiográfica. Todo considerado, éste es el factor más importante en la reducción de la fístula broncopleural y la úlcera del muñón bronquial.

Queda demostrada la cordura de esa filosofía por haberse tenido 105 casos consecutivos y mantenidos en observación más de un año de resección pulmonar sin ninguna complicación del muñón bronquial.

RESUME

La bronche tuberculeuse "quiescente": Etude de ses implications en chirurgie

Exposé des conceptions sur la trachéo-bronchite tuberculeuse. Il y a trois phases distinctes: a) l'ère pré-streptomycine—un problème inquiétant est posé; b) début de l'ère streptomycine—optimisme?; c) l'ère contemporaine—renaissance du problème inquiétant.

Les auteurs notent la tendance de la chimiothérapie à produire la cicatrisation superficielle de la bronche par épithélialisation, sans modification appréciable des lésions de la sous-muqueuse et de la paroi.

Les difficultés techniques générales exceptées, c'est l'état de la bronche tuberculisée au moment où elle est sectionnée qui est le facteur principal de la cicatrisation ou du défaut de cicatrisation. En pratique, l'état de la bronche peut être déterminé par un spécialiste en bronchoscopie. La conception de la bronche "quiescente" est exposée. Bien que, à l'egard de la tuberculose cette bronche ne soit pas "guérie", la guérison chirurgicale sera acquise après sectionnement. L'aspect bronchoscopique est décrit ainsi que diverses méthodes thérapeutiques.

Les auteurs suggèrent de considérer la bronche "quiescente" comme un *quatrième critère* dans la détermination de l'objectif de l'exérèse au même titre que l'oblitération cavitaire, la négativation des crachats et la stabilisation radiologique. Ceci est indubitablement le facteur le plus important pour la suppression de la fistule broncho- pleurale et de l'ulcération du moignon bronchique.

La sagesse de cette conception est démontrée par le succès de la résection pulmonaire sans complications au niveau du moignon bronchique, dans 105 cas consécutifs suivis pendant plus d'un an.

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PREDICTION OF RELAPSE IN PULMONARY TUBERCULOSIS[1]

An Application of Discriminant Analysis

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INTRODUCTION

The purpose of this paper is to explore the application of an actuarial prediction scheme to a medical problem. The all too common practice of accepting the generalizations of statistics as "facts" has led many to regard statistics with suspicion. To be sure, statistics does over-simplify, and the writers would be the first to agree that no exact forecast of the future can be made from the statistical analysis of data. It sometimes happens, however, that data are collected which, by themselves, yield neither information nor theory, and here statistics may be of some help. By the very fact of its over-simplification, statistics makes it possible to extract from the data some sort of system that in turn serves as a guidepost to areas in which further research may be fruitful. If it is kept in mind, therefore, that statistics is not an answer, but only a tool to aid in the search for scientific principles, it may be that statistical methods which have proved practicable in other fields will, upon trial, also prove useful in medicine.

In this paper is reported an actual trial of the statistical method of discriminant analysis with data collected from approximately 1,200 tuberculous patients, the outcome of whose disease was known. The mathematical aspects of the method are presented in detail in the "Materials and Methods" section, below.

* * * *

The method is essentially a simple one. To reduce it to its most elementary form, one may imagine a problem in which a flower is to be classified as one of two species. The only difference between the two species is the length of the petals. It has been found, by measuring, that the average petal of species *A* is 3 cm. in length; and of species *B*, 5 cm. Thus, it is supposed that 4 cm. is the separating, or critical score and, since the unclassified flower has a petal 4.2 cm. long, it is categorized as species *B*.

However, the process is complicated a bit more by the fact that an "average" score is only a mid-point, which does not reveal anything about the actual distribution or spread of scores. Suppose, for example, that none of the species *B* flowers which were measured had petal lengths farther away from the average than one-half centimeter. In other words, most of them fell between 4.5 and 5.5 cm. Species *A*, on the other hand, had a wide distribution, ranging from 2 to 4 cm. This would mean that a more accurate critical score would fall around 4.25 cm., rather than 4 cm., which would place the unknown flower with species *A* instead of *B*.

The original problem from which this method was developed was actually flower discrimination; Fisher (1), in 1935, used measurements of two species of iris to determine

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a critical score. But four measurements were used: petal length and width and sepal length and width. In the case of more than one variable, we have an additional problem: Does each of the variables have the same importance? If each does, the sum of the four measurements on each flower can be treated as a single composite variable. The critical score can then be determined in exactly the same way as before. That is, one first computes the mean composite score for each of the two species, which is of course the same thing as adding the means of the four original variables for each species. Then, if the composite variable happens to have the same spread in the two species, the critical score will simply be the mid-point between the two composite means. If the spreads are different, then the distance between the two composite means is partitioned in proportion to the two spread-magnitudes, and the partitioning point will represent the critical score.

However, the four variables usually do not have equal importance. For instance, the width of petals in species *A* may cover almost the same range for species *B*, with averages which are very close. In that case, it is difficult to differentiate the two species on the basis of petal width alone. Petal length, however, may be so distinct between the two species, with very little overlap in the respective ranges of measurements, that it is almost possible to base the decision as to species entirely on this variable. Petal length, in other words, has a higher correlation with membership in the different species and, in order to get full advantage of this high correlation, we assign a larger "weight" to petal length than to petal width. Similarly, weights are assigned to sepal length and sepal width in accordance with their relative importance in differentiating the two species. With a different weight multiplied to each variable, a weighted sum of the four variables is obtained in which the relative importance has been balanced.

This weighted sum is used as a composite variable, and the process is continued as before, getting the two species-means of the composite variable and determining the critical score which serves as the simple guidepost for any unclassified flower (of one or the other of the two species): if its weighted composite score falls below the critical score, it belongs to species *A*; if above it, to species *B*. The main problem, therefore, is how to determine the *optimal* weights for the several variables used as discriminators. It is to this problem that Fisher's technique of discriminant analysis gives an answer. And the composite variable obtained as the weighted sum using these optimal weights is called the linear discriminant function.

In the present study, there are 13 discriminator variables involved in the patients with tuberculosis, including sex, age, various data on the state of the disease on admission to the hospital and on discharge, length of hospitalization, type of treatment, et cetera. Whether the patient was well or relapsed by a certain date is the fourteenth variable, and this was used as the criterion by which to separate the sample into two groups, the "*well*" and the "*relapsed*."

The correlations of the 13 discriminator variables with one another and with the final outcome, the factor of *well* or *relapsed* (*see* table 3), give a rough indication of how much each variable contributed to the outcome and, therefore, how much weight should be given to it.

Following the computational procedure briefly described under "Materials and Methods," the linear discriminant function was computed. Then, the mean discriminant scores for the "well" group and the "relapsed" group were obtained, and the critical point for discrimination was determined by finding the mid-point between the two scores in terms of standard deviation units.

Once this critical score has been determined from the information about known cases, it may be useful as an indication for an unknown case. The critical score is not an absolute dividing line and, of course, the predictions will not be 100 per cent correct. As it was, a tryout on a sample of 300 cases yielded correct "predictions" for 220 cases, or 73.3 per cent of the group. While this may be a somewhat smaller percentage than might be aspired to in medical prognosis, it nevertheless seems to indicate that further investigations along these lines may prove fruitful. Several ways in which gains might be made in the accuracy of predictions are suggested in the "Discussion" below.

As a matter of incidental interest, it may be pointed out that discriminant analysis has been used in physical anthropology (2–4), clinical psychology (5–7), and educational research (8–11). Also worth mentioning is the excellent expository monograph on discriminant analysis and related statistical techniques recently written by Hodges (12), a researcher in the field of aviation medicine.

MATERIALS AND METHODS

The materials used in this study are the records of 1,259 patients with the adult type of pulmonary tuberculosis who were discharged from Glen Lake Sanatorium in the period from 1948 through 1953. Consequently, the post-discharge information covers periods from eleven months through six years.

A preliminary study using tests of statistical significance was conducted for isolating the variables that seemed relevant for the prediction of relapse. These were taken from among the mass of information available at the time of a patient's discharge. On the basis of this study, which was reported in a separate paper by Oyama (13), the following 13 variables were selected for use in the present discriminant analysis.

With the description of each variable is shown the numerical coding. Some of the variables, e.g., type of discharge, are categorical rather than quantitative; but their numerical coding is still justified because the preliminary study revealed a rough ordering of the categories. The categories of X_2 and X_6 were based on *Diagnostic Standards* published by the National Tuberculosis Association in 1950 (14).

X_1 = Sex: male = 1; female = 2

X_2 = Stage of disease on admission: minimal = 1; moderately advanced = 2; far advanced = 3

X_3 = Unilateral or bilateral pulmonary lesions: unilateral = 1; bilateral = 2

X_4 = Age on admission: 10–19 years = 1; 20–29 years = 2; . . . 70–79 years = 7

X_5 = Type of discharge: recommended = 1; acquiesced to = 2; with consent = 3; against advice = 4; transferred = 5; disciplinary = 6; deserted = 7

X_6 = Condition on discharge: inactive = 1; arrested (IV) = 2; arrested (III) = 3; active improved = 4; active unimproved = 5

X_7 = Bacteriologic findings on admission: no sputum available for culture of tubercle bacilli = 1; cultures for tubercle bacilli negative = 2; cultures for tubercle bacilli positive = 3; negative on microscopy but no cultures obtained = 4; sputum positive on microscopy for tubercle bacilli = 5

X_8 = Bacteriologic findings on discharge: more than twelve consecutive cultures for tubercle bacilli negative for at least one year = 0; more than six consecutive cultures for tubercle bacilli negative for six months = 1; fewer than six con-

secutive cultures for tubercle bacilli negative for six months = 2; occasional culture for tubercle bacilli positive for six months = 3; culture for tubercle bacilli consecutively positive but specimens negative on microscopy for six months = 4; negative on microscopy but no cultures obtained for six months = 5; sputum positive for tubercle bacilli on microscopy = 6; no sputum available for bacteriologic examination = 7

X_9 = Presence or absence of cavity on admission: no cavity = 0; unilateral cavity = 1; bilateral cavity = 2

X_{10} = Presence or absence of cavity on discharge: same coding as X_9

X_{11} = Length of hospitalization: 0–6 months = 1; 6–12 months = 2; 1–2 years = 3; 2–3 years = 4; 3–4 years = 5; 4–5 years = 6; more than 5 years = 7

X_{12} = Regimens of chemotherapy: streptomycin-isoniazid-PAS or streptomycin-isoniazid = 1; streptomycin-PAS = 2; isoniazid-PAS or isoniazid alone = 3; streptomycin alone or PAS alone = 4

X_{13} = Type of treatment: lung resection in combination with chemotherapy = 1; long-term chemotherapy (more than one year) = 2; collapse = 3; short-term chemotherapy (less than one year) = 4; bed rest only = 5

Approximately 1,200 of the 1,259 records were found to contain complete information on the above 13 variables. These cases were processed in the manner described hereunder.

First of all, each case was designated as "relapsed" or "well" according to whether the patient did or did not suffer a relapse of pulmonary tuberculosis as of November, 1954. (For further details such as the definition or criterion of relapse employed, see the paper by Oyama (13).) Eight hundred and fifty-two cases were thus labeled "well" and 348 "relapsed."

The next step was to divide the entire sample into subsamples: one, the normative sample on which to compute the discriminant function; and the other, the check sample to be used for empirical validation of the computed function. Since a larger number is advisable in the normative sample to ensure greater stability of the discriminant function, it was decided to have 900 cases in that sample and 300 in the check sample.

Also, in order that the two samples might be as comparable with each other as possible in terms of relative frequency of "well" and "relapsed" cases, as well as in the sex ratio, the selecting-out of the 300 check-sample cases was not done by pure random sampling, but rather by the stratified (or quota) sampling scheme. That is to say, the 1,200 cases were separated into four groups—male patients who were "well," female patients who were "well," male "relapsed," and female "relapsed"—and one-fourth of each group was selected at random to go into the check sample. The constitutions of the two subsamples thus formed are as shown in table 1.

To give some idea of the extent to which the "well" and "relapsed" cases differ with respect to the 13 predictor variables described above, the means of the two groups on each of these variables are shown in table 2. Also shown there are the means and standard deviations for the entire normative sample.

Outline of Computational Procedure

Since it is beyond the scope of this paper to describe the actual computational procedure of discriminant analysis, we will give only a very brief outline here. Those who are interested

TABLE 1

COMPOSITIONS, IN TERMS OF "WELL" AND "RELAPSED" CASES, AND OF SEX, OF THE NORMATIVE AND CHECK SAMPLES

	"Well"	"Relapsed"			"Well"	"Relapsed"	
Male	339	188	527	Male	113	63	176
Female	300	73	373	Female	100	24	124
	639	261	900		213	87	300
	Normative Sample				Check Sample		

TABLE 2

MEANS OF THE 13 VARIABLES IN THE "WELL" AND "RELAPSED" GROUPS AND THE ENTIRE NORMATIVE SAMPLE, WITH STANDARD DEVIATIONS SHOWN ONLY FOR THE ENTIRE SAMPLE

Variable	"Well" Group (N = 639)	"Relapsed" Group (N = 261)	Entire Normative Sample (N = 900)	
			Mean	Standard Deviation
X_1	1.47	1.27	1.41	0.49
X_2	2.23	2.41	2.28	0.64
X_3	1.64	1.82	1.69	0.46
X_4	3.54	4.12	3.70	1.55
X_5	2.63	3.71	2.94	2.03
X_6	2.50	3.61	2.82	1.45
X_7	3.38	3.74	3.48	1.26
X_8	1.81	3.23	2.22	1.83
X_9	0.73	0.82	0.76	0.67
X_{10}	0.08	0.44	0.18	0.50
X_{11}	2.95	2.79	2.91	1.27
X_{12}	2.41	2.86	2.51	1.08
X_{13}	3.34	4.12	3.58	1.46

in making practical applications of the technique are referred to texts by Fisher (15), Hoel (16), and Mather (17).

When only two groups are to be discriminated between, as in the present problem, the discriminant function is essentially the same thing as the multiple regression equation of a dummy variate (designating the groups) on the predictor variables.[3] Therefore, X_{14} is defined to take the values 1 and 2 for the two groups respectively. That is, each of the 639 patients in the normative sample who was "well" is given the value $X_{14} = 1$, and each of the 261 "relapsed" cases among the normative sample is assigned $X_{14} = 2$.

The product-moment correlation coefficient between each pair of variables is then computed by taking each one of the 14 (13 predictor and one criterion) variables in combination with the remaining 13. There are thus 91 [= (13)(14)/2] distinct correlation coefficients, which are shown in table 3 in the form of a symmetrical matrix with 182 off-diagonal elements.

In accordance with multiple regression theory, these correlation coefficients determine a set of 13 linear equations whose solution gives the set of 13 partial regression weights to be applied to the 13 predictor variables in standardized form. The rule for constructing

[3] For problems involving more than two groups, the computational procedure is different. Those interested may consult reference (18).

TABLE 3

COEFFICIENTS OF CORRELATION BETWEEN 13 PREDICTORS AND ONE CRITERION (GROUP-MEMBERSHIP) VARIABLE

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1		−0.061	−0.125	−0.298	−0.172	−0.076	−0.078	−0.089	−0.060	−0.098	0.026	0.071	−0.139	−0.178
2	−0.061		0.385	0.115	0.112	0.182	0.399	0.117	0.374	0.234	0.175	0.181	−0.082	0.128
3	−0.125	0.385		0.216	0.103	0.106	0.163	0.080	0.270	0.191	0.076	0.083	0.035	0.174
4	−0.298	0.115	0.216		0.057	0.125	0.035	0.151	0.001	0.167	−0.130	−0.125	0.256	0.170
5	−0.172	0.112	0.103	0.057		0.533	0.159	0.195	0.119	0.246	−0.202	−0.124	0.019	0.242
6	−0.076	0.152	0.106	0.125	0.533		0.206	0.344	0.178	0.381	−0.314	0.160	0.208	0.348
7	−0.078	0.399	0.163	0.035	0.159	0.206		0.168	0.424	0.172	0.295	0.095	−0.183	0.130
8	−0.089	0.117	0.080	0.151	0.195	0.344	0.168		0.044	0.340	−0.095	0.258	0.278	0.354
9	−0.060	0.374	0.270	0.001	0.119	0.178	0.424	0.044		0.470	0.265	0.001	−0.281	0.059
10	−0.098	0.234	0.191	0.167	0.246	0.381	0.172	0.340	0.470		−0.115	0.059	0.191	0.326
11	0.026	0.175	0.076	−0.130	−0.202	−0.314	0.295	−0.095	0.265	−0.115		0.177	−0.275	−0.059
12	0.071	0.181	0.053	−0.125	−0.124	0.160	0.095	0.258	0.001	0.059	0.177		0.406	0.189
13	−0.139	−0.082	0.035	0.256	0.019	0.208	−0.183	0.278	−0.281	0.191	−0.275	0.406		0.248
14	−0.178	0.128	0.174	0.170	0.242	0.348	0.130	0.354	0.059	0.326	−0.059	0.189	0.248	

TABLE 4

BETA-WEIGHTS, B-WEIGHTS, AND DISCRIMINANT WEIGHTS FOR THE 13 VARIABLES

Variable	Beta-Weight (β_i)	B-Weight (B_i)	Discriminant Weight (V_i)
X_1	−0.008	−0.001	−0.49
X_2	−0.011	−0.008	−0.04
X_3	0.096	0.095	0.51
X_4	0.039	0.011	0.06
X_5	0.092	0.021	0.11
X_6	0.164	0.052	0.28
X_7	0.032	0.012	0.06
X_8	0.159	0.039	0.21
X_9	−0.133	−0.089	−0.48
X_{10}	0.204	0.186	1.00
X_{11}	0.087	0.031	0.17
X_{12}	0.088	0.037	0.20
X_{13}	0.058	0.018	0.10

these equations may be inferred from the first two and the thirteenth equations, shown below:

Equation 1:

$$\begin{aligned}
\beta_1 - 0.061\beta_2 - 0.125\beta_3 - 0.298\beta_4 - \cdots + 0.071\beta_{12} - 0.139\beta_{13} &= -0.178 \\
-0.061\beta_1 + \beta_2 + 0.384\beta_3 + 0.115\beta_4 + \cdots + 0.181\beta_{12} - 0.082\beta_{13} &= 0.128 \\
\vdots \quad\quad \vdots \quad\quad \vdots \quad\quad \vdots \quad\quad \vdots \quad\quad \vdots \quad\quad \vdots \quad\quad \vdots \quad\quad \vdots & \\
-0.139\beta_1 - 0.082\beta_2 + 0.035\beta_3 + 0.256\beta_4 + \cdots + 0.406\beta_{12} + \beta_{13} &= 0.248
\end{aligned}$$

The resulting standard partial regression weights (or beta-weights, as they are usually called) are shown in the first column of table 4. The magnitude of each beta-weight indicates the relative importance of the corresponding variable within the set of 13 predictors.

The next step is to transform the weights into raw-score partial regression weights, or the B-weights. This is done by multiplying all the beta-weights by the standard deviation of the criterion variable, X_{14} (which was 0.454), and then dividing each such product by the standard deviation of the corresponding predictor variable; the 13 standard deviations are shown in the last column of table 2. The resulting B-weights are shown in the second column of table 4.

The final computational step is taken just as a matter of convenience, in order to have the largest weight equal to unity. This is possible because discriminant function weights need only be determined up to an arbitrary proportionality factor. Therefore, one divides each B-weight by 0.186, which happens to be the value of B_{10}, the largest B-weight. The results, shown in the last column of table 4, are the conventionalized discriminant function weights, $V_1, V_2, \ldots V_{12}$.

Using these V-weights as the combination coefficients, the desired discriminant function is constructed as shown in Equation 2.

$$Y = -0.49X_1 - 0.04X_2 + 0.51X_3 + 0.06X_4 + 0.11X_5 + 0.28X_6 + 0.06X_7 + 0.21X_8 - 0.48X_9 + 1.00X_{10} + 0.17X_{11} + 0.20X_{12} + 0.10X_{13} + 0.74$$

The constant term, 0.74, was added merely to avoid having a negative value for any individual's discriminant function score, and also (incidentally) to make the mean of Y for the entire normative sample equal to an integer. As may be verified by substituting, in turn, the three sets of means shown in table 2 for $X_1, X_2, X_3, \ldots X_{13}$, in Equation 2, the means of Y in the "well" group, the "relapsed" group, and the total normative sample are, respectively,

$$\bar{Y}_w = 3.58 \qquad \bar{Y}_r = 5.00 \qquad \bar{Y}_{\text{tot.}} = 4.00$$

While the foregoing completes the computation, as such, of the discriminant function, there still remains the important matter of establishing the *decision rule*. That is to say, a critical value of the discriminant function must be determined so that, when using Equation 2 for the purpose of forecasting whether a newly discharged patient will or will not suffer relapse, one will predict "yes" if his discriminant function score exceeds this value, and predict "no", i.e., that he will be "well," if his score falls short of the critical score.

In the statistical theory of classification there are several ways of establishing a decision rule. Alternately, it might be more precise to say that there are several different considerations to be taken into account with respect to the possible damage incurred by making a decision which later proves to be an erroneous one; and different judgments concerning the relative seriousness of various (in the two-group case, just two) alternative types of misdecisions lead us to adopt different decision rules.

For instance, in the present case, it may be deemed more disastrous to predict "no relapse" for a person who in fact later comes to suffer relapse than conversely to predict relapse for a person (and hence, presumably, discourage him from becoming discharged) when actually he may not have suffered relapse after all even if permitted to leave the hospital.

Again, it may, in certain situations, be advisable to aim at minimizing the over-all *number* of misdecisions; in yet other cases the objective might be to minimize the *sum* of the proportions of misdecisions of various types (which would not be equal to the *over-all* proportion unless the number of persons in the alternative groups are approximately equal).

Some of the different decision rules appropriate for one or another of the various alternative long-run objectives mentioned above are as follows:

Rule 1: Take the straight arithmetic mean of $\bar{Y}_w$ and $\bar{Y}_r$ as the critical value. That is, if a newly discharged patient's Y-score as computed from Equation 2 is *less* than (3.58 + 5.00)/2 (which is 4.29), predict that he will *not* suffer relapse;

Rule 2: Take the mean Y-score of the entire normative sample, $\bar{Y}_{\text{tot.}} = 4.00$ as the critical value.

Rule 3: Take as the critical value that value which is equidistant in standard deviation units from $\bar{Y}_w$ and $\bar{Y}_r$. In the case at hand this value was found to be 4.36.

After various considerations, especially the observation that the "well" and "relapsed" groups had rather different spreads in the discriminant scores, it was decided to adopt *Rule 3* in the present study. Theoretically, this rule minimizes the over-all number of misdecisions in the long run, which, in view of the (happily!) vast discrepancy in number

TABLE 5

OUTCOME OF PREDICTIONS OF RELAPSE OR NONRELAPSE ON A CHECK SAMPLE OF 300 CASES

Prediction by Equation 2	Actual Status		Total
	Did not relapse	Did relapse	
Will not relapse	163	30	193
Will relapse	50	57	107
Total	213	87	300

between the "well" and "relapsed" cases, seems preferable to minimizing either the over-all proportion or the sum of proportions of the two types of misdecision.

OBSERVATIONS

Tryout on Check Sample

The discriminant function has been computed, viz., Equation 2, which is reproduced below for convenience. The critical discriminant score, 4.36, has also been established. In practical application, the items of information on a patient are sorted into the 13 variables previously listed, and are converted into the appropriate code values of $X_1, X_2, \cdots X_{13}$. These values are substituted in Equation 2 in order to compute the discriminant score Y for this patient. If this score exceeds the critical score of 4.36, it is predicted that this patient will suffer relapse; otherwise, that he will not.

Equation 2:

$$Y = -0.49X_1 - 0.04X_2 + 0.51X_3 + 0.06X_4 + 0.11X_5 + 0.28X_6 + 0.06X_7 + 0.21X_8 - 0.48X_9 + 1.00X_{10} + 0.17X_{11} + 0.20X_{12} + 0.10X_{13} + 0.74$$

At this point an attempt was made to see how well this prediction scheme could be expected to work in practice. A check sample of 300 cases selected from among the total group of 1,200 cases was reserved for this purpose, that is, these 300 cases were "thrown out", not used at all, so far as the construction of the discriminant function and establishing of the critical score were concerned. The check sample may therefore be regarded as a reasonable facsimile of a future group of patients being considered for discharge from the hospital. The discriminant score Y was computed for each of these 300 cases in accordance with Equation 2. A "prediction" of "relapse" or "well" was made for each patient dependent on whether his or her Y-score exceeded or fell short of the critical score 4.36. The outcome thus "predicted" for each of the 300 cases was then compared with the actual status of that patient as of November, 1954. In table 5 is shown the extent to which the "predictions" were successful.

From these results, it may be seen that, of the 213 patients who were well, 163 could have been so predicted on the basis of information available at the time of the patient's discharge by using Equation 2 and the critical score 4.36; of the 87 who did suffer relapse, 57 would have received this unfavorable forecast at the time of discharge from the hospital.

Viewing table 5 in the other way, it may be seen that the use of the prediction scheme would have led us to predict nonrelapse for 193 patients, and this prediction would have been true for 163 of these 193. On the other hand, of the 107 for whom we would have forecast relapse, only 57 would actually have shown relapse as of November, 1954. Taken as a whole, the percentage of correct predictions is (163 + 57)/300, or 73.3 per cent.

Illustrative Examples

In order to illustrate in full detail the practical application of the prediction scheme, the procedure is described for four typical cases chosen from the check sample.

Example 1 (No. 10458): A white girl (hence $X_1 = 2$) was admitted to the hospital in July, 1948, with minimal ($X_2 = 1$) unilateral pulmonary tuberculosis ($X_3 = 1$). The patient, aged nineteen years on admission ($X_4 = 1$), was given a recommended discharge from the hospital ($X_5 = 1$) in November, 1950. Her condition on discharge from the hospital was "arrested (IV)" ($X_6 = 2$); the culture of gastric contents revealed tubercle bacilli on admission ($X_7 = 3$). Since ten gastric cultures were negative for tubercle bacilli throughout 1949, no sputum was available for bacteriologic examination during the last six months ($X_8 = 7$). The chest roentgenogram disclosed no cavitation on admission ($X_9 = 0$) or on discharge ($X_{10} = 0$). During her two years and three months' hospitalization ($X_{11} = 4$), streptomycin, 1 gm. daily ($X_{12} = 4$), was administered for forty-one days ($X_{13} = 4$).

On the basis of the above data, would one *predict* that the patient will suffer a relapse or not? Computation of her discriminant score Y in accordance with Equation 2 yields:

$$Y = -0.49x2 - 0.04x1 + 0.51x1 + 0.06x1 + 0.11x1 + 0.28x2 + 0.06x3 + 0.21x7 - 0.48x0 + 1.00x0 + 0.17x4 + 0.20x4 + 0.10x4 + 0.74 = 4.49.$$

The patient's discriminant score, 4.49, is *greater than the critical score*, 4.36. Accordingly, the prediction is that she *will* suffer relapse. Actually, the patient was readmitted to the hospital in March, 1953, because of a recurrence.

Example 2 (No. 10664): A white man ($X_1 = 1$), aged thirty-five ($X_4 = 3$), with the diagnosis of far advanced ($X_2 = 3$) bilateral pulmonary tuberculosis ($X_3 = 2$), was admitted to the hospital in September, 1951. He received "with consent" discharge from the hospital ($X_5 = 3$) in June, 1952, because of the "active improved" condition ($X_6 = 4$). The sputum, which was positive for tubercle bacilli on admission to the hospital ($X_7 = 5$), revealed occasionally positive cultures for tubercle bacilli during his last six months in the hospital ($X_8 = 3$). The chest roentgenogram showed no evidence of cavitation on admission ($X_9 = 0$) or on discharge ($X_{10} = 0$). During nine months' hospitalization ($X_{11} = 2$), he was treated with streptomycin, 1 gm. twice weekly, and PAS, 12 gm. daily ($X_{12} = 2$), for eight months ($X_{13} = 4$).

Equation 2 yields the following discriminant score:

$$Y = -0.49x1 - 0.04x3 + 0.51x2 + 0.06x3 + 0.11x3 + 0.28x4 + 0.06x5 + 0.21x3 - 0.48x0 + 1.00x0 + 0.17x2 + 0.20x2 + 0.10x4 + 0.74 = 4.85$$

TABLE 6

DATA AND THE RESULTING DIMCRIMINANT SCORES FOR TWO PATIENTS

Item	Specification and Numerical Code		Variable
	Case No. 10046	Case No. 11132	
Sex	Female = 2	Male = 1	X_1
Stage of disease on admission	Far advanced = 3	Far advanced = 3	X_2
Unilateral or bilateral pulmonary lesions	Bilateral = 2	Bilateral = 2	X_3
Age on admission	32-years-old = 3	35-years-old = 3	X_4
Type of discharge	Against advice = 4	Recommended = 1	X_5
Condition on discharge	Active = 5	Inactive = 1	X_6
Bacteriologic findings on admission	Positive sputum on microscopy = 5	Positive sputum on microscopy = 5	X_7
Bacteriologic findings on discharge	Fewer than 6 cultures negative = 2	Fewer than 6 cultures negative = 2	X_8
Cavitation on admission	Bilateral = 2	None = 0	X_9
Cavitation on discharge	None = 0	None = 0	X_{10}
Length of hospitalization	1 year 1 month = 3	1 year 6 months = 3	X_{11}
Regimens of chemotherapy	Streptomycin alone = 4	Streptomycin-PAS = 2	X_{12}
Type of treatment	Chemotherapy for 4 months = 4	Wedge resection = 1	X_{13}
Discriminant function score computed from Equation 2	Y = 4.15	Y = 3.87	
Prediction	Well	Well	

Since this value is greater than 4.36, the prediction is that the patient will suffer a relapse. He did suffer a relapse, and was readmitted in March, 1953, in agreement with the prediction.

Examples 3 and 4: Data for the remaining two cases chosen for exemplification are shown in tabular form (table 6) for brevity.

For patient No. 10046, Equation 2 yields:

$$Y = -0.49x2 - 0.04x3 + 0.51x2 + 0.06x3 + 0.11x4 + 0.28x5 + 0.06x5 + 0.21x2 - 0.48x2 + 1.00x0 + 0.17x3 + 0.20x4 + 0.10x4 + 0.74 = 4.15$$

as her discriminant score. Since this value is less than the critical value, 4.36, the prediction is that she will *not* suffer a relapse. According to the records dating from her discharge in July, 1948, through November, 1954, the prediction remained correct. Moreover, this follow-up period is probably long enough to bar any future reversal.

Similarly, for patient No. 11132, substitution in Equation 2 of the appropriate values for X_1 through X_{13} yields $Y = 3.87$ as his discriminant score. Since this value is smaller than 4.36, the prediction was again a favorable one. The patient's

readmission to the hospital in July, 1952, however, proved the prediction to have been incorrect in this case.

DISCUSSION

As mentioned at the outset of this paper, it is far from the intention of the writers to claim anything approaching infallibility for the prediction scheme developed and exemplified in the foregoing. It is hoped and believed, however, that the proposed method might be of some aid in objectifying the judgment of the wisdom of a patient's prospective discharge from the hospital; hence, in decreasing the frequency of reactivations of pulmonary tuberculosis.

Some possible ways in which the predictive efficiency of the discriminant function could be improved will now be considered. The most obvious way, of course, is to look for additional and more significant factors to be used as predictor variables. One such factor which immediately suggests itself is the socio-economic status of the patient: the type of life a patient leads once he is out of the hospital is, beyond doubt, an important factor influencing the chances of his suffering a relapse.

Apart from the scope of variables included, however, the present study had the limitation that the follow-up period varied in length from one patient to another. Ideally, this should be held constant at, say, five years—a period of time beyond which it is fairly safe to assume that, if a person has not suffered reactivation, it is unlikely that he ever will.

On the mathematical side, a possible way to improve the predictive efficiency beyond the 73.3 per cent that was attained would be to introduce suitable transformations for some of the variables. This is because some of the variables seem to have markedly skewed distributions, as evidenced by the fact that their standard deviations were of the same order of magnitude as the means (and in one case, X_{10}, even greater than the means). Such transformations mean including higher orders than the first in some of the variables, and hence would allow for relationships other than linear ones among them.

Despite the limitations of the present investigation implied by the above suggestions for improvement, the writers believe that, as an exploratory study, it has the merit of pointing out the possibilities for fruitful application of a statistical technique that seems hitherto to have remained relatively unnoticed by researchers in the medical field. If this paper helps to stimulate an interest in similar applications of discriminant analysis, it will have served its purpose.

SUMMARY

In this paper, the possibility of applying the statistical technique of discriminant analysis to the prediction of relapse in pulmonary tuberculosis was subjected to an actual trial. A linear discriminant function (Equation 2) was developed, and a critical score of 4.36 was established. If the data on a patient under consideration for discharge from the hospital are such that, following the procedure described in the paper, they yield a discriminant score greater than 4.36, it would be predicted that this patient would suffer a relapse. Predictions

on this basis were found to be borne out in 220 of 300 test cases. Possibilities for improving the predictive efficiency are discussed.

SUMARIO

Predicción de las Recidivas en la Tuberculosis Pulmonar: Aplicación del Análisis Discriminante

La posible aplicación de la técnica estadística del análisis discriminante a la predicción de las recidivas en la tuberculosis pulmonar fué objeto de una prueba real. Se elaboró una función discriminante lineal (Ecuación 2), estableciéndose una marca de 4.36. Si los datos obtenidos en un enfermo estudiado con mira a darlo de alta del hospital son tales que, conforme al procedimiento descrito en este trabajo, arrojan una marca discriminante superior a 4.36, cabe predecir que el enfermo experimentará una recidiva. Las predicciones formuladas a partir de esa base quedaron confirmadas en 220 de 300 casos estudiados. Discútense las posibilidades de mejorar el acierto en la predicción.

RESUME

Prédiction des rechutes dans la tuberculose pulmonaire: Application de l'analyse discriminante

Dans cet article, la possibilité d'appliquer la méthode statistique d'analyse discriminante à la prédiction des rechutes dans la tuberculose pulmonaire a été mise à l'épreuve. Une fonction discriminante linéaire (Equation 2) a été développée et un score critique de 4,36 a été établi. Lorsque les données obtenues chez un malade dont on envisage le départ de l'établissement sont telles que, suivant les procédés décrits dans cet article, on obtient un score discriminant supérieur à 4,36, la prédiction d'une rechute sera faite pour ce malade. Les prédictions énoncées sur cette base ont été confirmées dans 220 cas sur 300 cas-tests. Les possibilités d'accroître l'efficacité des prédictions sont commentées.

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PULMONARY NOCARDIOSIS

A Review with a Report of Seven Cases

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INTRODUCTION

Nocardiosis is an acute or chronic suppurative disease with a tendency to remissions and exacerbations which seems to have a special predilection for the lungs, brain, and subcutaneous tissue. Hematogenous spread may lead to abscesses in various parts of the body. Multiple draining sinuses may result, but are less common than in actinomycosis. Nocardiosis remains an infrequent and poorly recognized mycosis.

In 1888, Nocard described an acid-fast sporothrix, *Actinomyces forcincus*, as the etiologic agent in bovine farcy. Three years later, in 1891, Eppinger demonstrated an aerobic gram-positive acid-fast actinomyces in the pus from a meningitis and cerebral abscess. The organism was called *Cladothrix asteroides*. This was the first human case recorded.

Twenty-six cases of nocardiosis were collected from the literature by Henrici and Gardner in 1921. Twenty-two of these cases were fatal. Forty-four cases of pulmonary nocardiosis have been found in the literature.

MYCOLOGY

The nocardia appear microscopically as gram-positive bacilliform filaments which may become fragmented and resemble diphtheroids. They are often referred to as bacteria-like fungi. These organisms were shown by Claypole in 1913 to have feeble acid-fast staining properties. Twelve species display acid-fast properties. They may gradually lose their acid-fast characteristics following repeated transfers on artificial media. This is particularly true when acid alcohol is used to decolorize as a part of the Ziehl-Neelsen technique. This condition may be offset, however, by the use of a weak solution of sulfuric acid even though continued transfers have been made. Different strains show varied degrees of acid-fast staining properties. Ordinarily, the older the culture, the more irregular is this characteristic.

Forty-two species of nocardia have been recognized. The most common of these is *N. asteroides* which is the etiologic agent in the 7 cases reported in this paper. This fungus is a member of the family *Actinomycetaceae* which represents a transitional group of organisms between the true bacteria (Eubacteriales) and the true fungi (Eumycetes). Two genera are found in this family, namely, the actinomyces which are anaerobic, and the nocardia which are aerobic.

Nocardiosis is practically never diagnosed until appropriate stains and special cultures are utilized. The organisms grow rather well on the ordinary media under aerobic conditions, but usually seem to prefer an alkaline medium. Growth

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may occur when planted on blood glucose agar or Sabouraud's glucose agar within three to six days in a temperature range of 68°F. (room temperature) to 98.6°F. (body temperature).

The nocardial colonies often assume a mycelial ball-like appearance resembling a star. At times, they look dry and wrinkled, and may lie free on the surface as a pellicle. They do not ferment carbohydrates; and do not produce indole, hydrogen sulfide, or ammonia.

Granules varying in color from yellow to orange are sometimes expectorated in the sputum. These granules represent masses of intertwining, branching mycelial filaments 0.25 to 1 micron in diameter.

World-wide in distribution and regarded as saprophytic in nature, the mycelia are usually isolated from the soil, but may be found on grasses, straw, and grains. It is doubtful if these fungi are truly pathogenic. If a minor niche or excoriation of an epithelial surface occurs, however, the organisms may enter the body. It is thought, moreover, that the infection may gain entrance by skin penetration, inhalation, or ingestion.

It is interesting to note that the pathogenicity for guinea pigs and rabbits is not constant in virulence studies. If the organisms are injected intravenously or intraperitoneally, miliary abscesses develop in the viscera or peritoneum. Such animals may die in seven to fourteen days.

Mere localized disease or abscess formation may develop with subsequent healing or spontaneous drainage when subcutaneous or intramuscular inoculations are administered. No complement-fixing antibodies or agglutinins have been demonstrated in man.

PATHOLOGY

In pulmonary nocardiosis, a caseous bronchopneumonia with subsequent necrosis and cavitation may occur. Extension of the process to the pleura with formation of an empyema may be observed. In the early stages, there is usually a suppurative lobular pneumonia. The lesions may occur in any lobe, but are more common in the lower lobes. Involvement of the peribronchial lymph nodes may occur early.

Microscopic observation of the pulmonary lesion reveals a center composed of necrotic material surrounded by dense cellular fibrous tissue, fibrin, leukocytes, plasma cells, and macrophages. Branching mycelia are usually scattered throughout the tissue.

SYMPTOMATOLOGY AND DIAGNOSIS

The symptoms and physical signs of pulmonary nocardiosis are those found in an acute or chronic pneumonitis or those due to spread to other organs. The onset may be sudden with high fever, chills, cough, pain in the chest, and prostration. On the other hand, there may be an insidious onset with anorexia, malaise, weight loss, night sweats, fatigue, afternoon fever, and a chronic cough productive of foul greenish mucoid sputum. Hemoptysis seldom occurs. Small orange or yellow granules may be expectorated at times. Hepatomegaly and splenomegaly represent late stages of dissemination.

Rarely, the disease may remain dormant and asymptomatic for long periods of time. It may flare up acutely and then be followed by a dormant course. Some patients die within two weeks after the onset of symptoms while, in others, the disease may remain active for as long as three years. The average duration has been found to be approximately six months.

The diagnosis is made by demonstrating the presence of the nocardia in the sputum by the use of appropriate stains and cultures. A cutaneous test for the diagnosis of actinomycotic mycetoma caused by *N. brasiliensis* is advanced by Gonzalez. On the whole, cutaneous tests have been inconstant. Blood cultures positive for nocardia are very unusual during life, but such a case, with eventual recovery of the patient, is reported by Kohn and others. A case of initial infection of the femur with subsequent embolic abscesses in multiple organs is reported by Cruz and Clancy. A most interesting finding in nocardiosis may be a very marked leukocytosis of 15,000 to 50,000 cells per cu. mm.

Roentgenograms of the chest may resemble those seen with any common infectious granuloma. As noted previously, the lesions are more common in the lower lobes, but may occur in any lobe. There is usually a small infiltration or there may be large lobular consolidations.

DIFFERENTIAL DIAGNOSIS

Pulmonary nocardiosis is often confused with pulmonary actinomycosis. Actinomycosis appears as a more distinctive chronic granulomatous suppurative process with induration or multiple draining sinuses. "Sulfur granules" are usually found sooner or later. Nocardiosis is more often found in the lower lobe. The usual causative agent, *A. bovis*, is anaerobic. *N. madurae* and *N. pelletieri* produce acute and chronic suppurative and granulomatous lesions very similar to actinomycosis.

Nocardiosis should be considered in any lesion of the lung resembling tuberculosis in which tubercle bacilli are not found. The pathologic changes may closely simulate the course of acute miliary tuberculosis. Since nocardia produce acid-fast bacilliform filaments, the microorganisms are easily confused with tubercle bacilli. McQuown believes that approximately 5 per cent of the patients in tuberculosis sanatoriums may have nocardiosis. Such patients could easily develop a superimposed tuberculosis. Incidentally, the marked leukocytosis may be an aid in differentiating tuberculosis or suspected pulmonary neoplasms which may frequently be a real diagnostic problem.

Other pulmonary mycoses such as blastomycosis, histoplasmosis, coccidioidomycosis, cryptococcosis, or sporotrichosis may be easily confused with nocardiosis. Sarcoidosis may resemble nocardiosis. Lung abscess, viral pneumonia, and tularemia must be considered in the differential diagnosis.

TREATMENT

Early diagnosis of pulmonary involvement with nocardia and the institution of appropriate treatment may mean the difference between survival or death. In animal protection tests against nocardiosis, Strauss and co-workers found that sodium sulfadiazine provided almost perfect protection, enabling 100 per cent

of infected mice to survive, while chlortetracycline, chloramphenicol, and nyvizone gave only partial protection. Actinomycosis may respond readily to penicillin or other antimicrobials, while nocardiosis responds to sulfadiazine alone or along with other antimicrobials.

At present, treatment with sulfadiazine and a companion drug, such as penicillin or tetracycline, seems to be the therapy of choice. Early lesions may respond to symptomatic care and antimicrobial therapy. In more severe cases, surgical intervention with adequate drainage or complete excision of the infected area may be necessary.

The disease was fatal except for a few isolated cases before the advent of therapy with sulfadiazine in 1944. The prognosis depends upon the length of time the disease has progressed and upon the extent of involvement.

PRESENT SERIES

Seven cases of pulmonary nocardiosis are herein presented. *Cases 1*, *2*, and *3* are considered to represent diagnostic problems. *Cases 4*, *5*, and *6* are considered to represent cases in which diagnosis seems secure; *Case 7* is presented as an example of a case with a classical onset of the disease.

Cases with Diagnostic Problems

Case 1. Pulmonary nocardiosis; nocardia abscess; syphilitic aortic aneurysm (September 15, 1953). The patient, O. C., was a 55-year-old white married housewife who was well until April, 1953, when she developed a "chest cold" which was untreated until June 9, 1953. At this time she developed a chronic cough productive of greenish sputum, wheezing, dyspnea, pain in the chest, temperature of 100° to 101°F., and had three or four chills. A chest roentgenogram revealed a consolidation of the left lower lobe. Treatment with oxytetracycline produced virtually no improvement. On June 28, 1953, a roentgenogram showed slight clearing of the process. On September 15, 1953, the patient began to notice hemoptysis. Her weight was declining rapidly at this time. It was noted that she had been found to have a serologic test positive for syphilis four years previously and had been treated with three courses of ten million units of penicillin. There was no family history of cancer or tuberculosis.

Physical examination revealed a malnourished woman who had a temperature of 101.4°F.; pulse, 86 per minute; respirations, 22 per minute. The trachea was felt in the mid-line. There were harsh excretory rhonchi anteriorly in the left mid-clavicular area near the fourth interspace. A loud systolic murmur was noted over the mitral area. The hemoglobin was 10 gm.; packed cell volume, 37 per cent; leukocyte count, 9,800 per cu. mm. with the differential count revealing 71 per cent polymorphonuclear leukocytes; and an erythrocyte sedimentation rate of 20 mm. Serologic tests for syphilis were positive. A cerebrospinal fluid Wasserman reaction was positive. Repeated sputum examinations revealed acid-fast organisms "resembling nocardia" by the Ziehl-Neelsen stain. No spirochetes or fusiform bacilli were demonstrated. A sputum culture was negative for *M. tuberculosis* at the end of eight weeks. A culture on Sabouraud's medium revealed *Nocardia asteroides*.

A chest film showed a large cavity in the upper portion of the left lower lobe posteriorly. Infiltration extended from the cavity inferiorly. There was widening of the mediastinum which could not be separated from the aorta and was found by sectional views and by fluoroscopic examination to project from the arch of the aorta. It was believed at that time that the stringy infiltration below the abscess area was suggestive of tuberculosis (figure 1). The possibility of carcinoma was also considered. The cutaneous reaction to tuberculin (OT 1:1,000) was positive, and the reaction to histoplasmin was negative. Extensive treat-

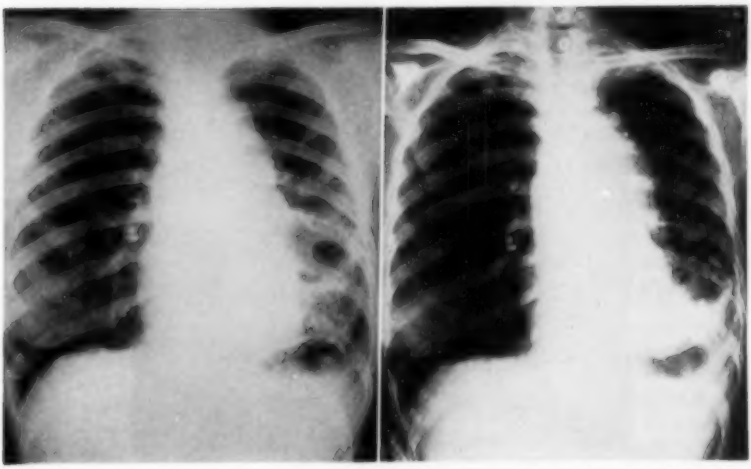

FIG. 1 (Left). *Case 1.* Roentgenogram of chest, taken on September 15, 1953, showing cavity in the upper portion of the left lower lobe.

FIG. 2 (Right). *Case 1.* Roentgenogram of the chest, taken on July 30, 1954, revealing much less infiltration in the left lower lobe.

ment with penicillin, chlortetracycline, and other antimicrobials produced no improvement. Sulfadiazine was begun and the fever gradually subsided.

A chest roentgenogram obtained on November 23, 1953, showed improvement in the density in the left lower lobe. On July 8, 1954, the patient's temperature was 99.6°F.; hemoglobin, 11.2 gm.; leukocyte count, 16,500 per cu. mm.; and erythrocyte sedimentation rate, 30 mm. A film of the chest showed the lung abscess to be less marked, with no definite cavity. On August 17, 1954, a sputum culture was still positive for *Nocardia asteroides*. On July 30, 1954, the aneurysm was visible on the roentgenogram. There was slight prominence of the left ventricle, and infiltration and thickened pleura were visualized in the left apex and left base (figure 2). On April 4, 1955, a chest roentgenogram showed fibrosis in the left apex and very slight infiltration in the left lower lobe. The temperature was 98.6°F. The sputum culture was still positive for *N. asteroides*. The leukocyte count was 8,600 per cu. mm.; the sedimentation rate was 12 mm. Treatment with sulfadiazine is being continued.

Comment: It would be easy to see that in a case of a saccular aneurysm of the descending aorta a post-stenotic abscess of the left lower lobe might be produced. However, on careful study this aneurysm seemed to extend from the upper arch of the aorta. Likewise, in this particular case, eight sputum specimens were found to be positive for *Nocardia asteroides* by culture and animal inoculation. In addition, a quantity of tissue was expectorated, examination of which revealed *Nocardia asteroides* in the tissue. Four sputum cultures were negative for tubercle bacilli.

Case 2 (October 10, 1954). The patient, D. R., was a sixteen-year-old white male student who had been a known asthmatic since the age of three, requiring symptomatic treatment, such as sprays and an occasional injection of epinephrine. A chest roentgenogram at the age of nine was said to have been normal. On September 11, 1954, the patient developed a

FIG. 3. *Cases 2* and *6*. *Nocardia asteroides* colonies on blood glucose agar.

high fever, general malaise, and cough productive of mucoid sputum; he had two chills. A roentgenogram of the chest was thought to reveal tuberculosis in the left apex. A series of eight sputum examinations revealed one with a few acid-fast organisms.

Upon first examination, in October, 1954, the patient was found to be well developed and well nourished, but with slight dyspnea. His temperature was 101.2°F.; pulse, 96 per minute; and respirations, 24 per minute. There was bogginess of the turbinates, a mild pharyngeal injection, and many sonorous rales throughout the chest. Asthmatic breath sounds were present in all areas. A few moist rales were audible in the left apex. The hemoglobin was 13.5 gm.; the leukocyte count, 26,200 per cu. mm., with 68 per cent polymorphonuclear leukocytes; the erythrocyte sedimentation rate was 32 mm.; the blood culture was sterile. A tuberculin test (OT 1:1,000) was negative.

A chest roentgenogram revealed an infiltration in the left apex suggestive of tuberculosis. A series of twelve sputum examinations revealed no tubercle bacilli, but did reveal acid-fast staining bacilliform fragments. Culture on blood glucose agar was positive for *Nocardia asteroides* (figure 3). Three cultures were negative for *M. tuberculosis* after eight weeks' incubation.

A diagnosis of pulmonary nocardiosis was made. The patient received sulfadiazine, starting on December 1, 1954, in a dosage of 1.0 gm. every four hours for one week, then, 1.0 gm. four times daily for four months. A chest roentgenogram on January 12, 1955, showed some clearing of the left apical shadow. The temperature had fallen to 99.0°F. On April 14, 1955, a chest film showed a marked decrease in the left apical lesion. The temperature was 98.4°F.; the leukocyte count was 8,800 per cu. mm. The erythrocyte sedimentation rate was 12 mm. There was no further cough. The sputum was negative for *Nocardia asteroides* by culture and animal inoculation. The bronchial asthma was quiescent. The lungs were clear to percussion and auscultation. Follow-up observation of the patient is continuing.

Comment: In this case, four cultures for *M. tuberculosis* were negative and the tuberculin and histoplasmin skin reactions were likewise negative. Six cultures were positive for *Nocardia asteroides* by culture and animal inoculation. In addition, the initial chemotherapy with chlortetracycline failed to result in any clearing of the lesion. It is believed that this represents a case of pulmonary

nocardiosis and not an instance of a bacterial process which resolved promptly with antimicrobial therapy and in which the nocardia happened to be an added finding in the sputum. Moreover, the very chronic nature of the process would favor this diagnosis.

Case 3 (December 14, 1953). W. S., a 55-year-old white married male warehouse foreman, was admitted to the hospital stating that approximately one month previously he had contracted a cold, characterized by sneezing, rhinitis, mild cough, and generalized malaise. Approximately two weeks before this time, he became worse, visited his local physician who gave him four injections of penicillin and prescribed tetracycline and acetylsalicylic acid. He had had fever as high as 104°F., and had had several chills. On admission, it was learned that for the past two days the temperature had been much higher and the patient seemed much worse. He was coughing and bringing up large black mucoid clots of sputum; but there had been no frank hemoptysis. Night sweats had been present for two or three weeks. There had been no known weight loss. The past history revealed excellent health throughout the patient's life. There was no family history of tuberculosis.

Physical examination revealed a well-developed and well-preserved man in moderate distress. The temperature was 102°F.; pulse, 120 per minute, respirations, 24 per minute. The throat was normal. The trachea was not deviated. The right lung anteriorly, superiorly, and inferiorly revealed moist crepitant rales on inspiration and expiration. There was dullness to percussion over the right upper portion of the chest anteriorly and posteriorly. No rales were heard in the left lung. The hemoglobin was 12 gm.; the leukocyte count, 10,250 per cu. mm. with a shift to the left; erythrocyte sedimentation rate, 18 mm.; routine agglutinations, negative; blood culture, sterile; and cold agglutinins, negative. Culture and smear for acid-fast bacilli were negative. The sputum smear revealed a mixed bacterial flora with gram-positive cocci predominant, a few gram-negative diplococci, and many gram-positive rods. Culture on blood glucose agar revealed *Nocardia asteroides*. A histoplasmin test and a tuberculin test (OT 1:1,000) were negative.

A chest roentgenogram upon admission revealed infiltration throughout the entire right upper lobe which had the appearance of a lobar pneumonia, but also suggested the possibility of a tuberculous pneumonia (figure 4). A repeat film one week later showed some clearing of the lesion. The patient was treated with parenteral fluids, erythromycin, and sulfadiazine, 1.0 gm. every four hours for two weeks, with marked subjective improvement and return of temperature to normal. On December 30, 1953, a chest film showed marked clearing of the infiltration in the right upper lobe. Sulfadiazine was continued, 1.0 gm. four times daily for eight months, without any urinary or hemopoietic abnormalities.

On January 20, 1954, a chest roentgenogram showed some increased markings in the right upper lobe, but no consolidation. On February 21, 1954, marked improvement was seen on the roentgenogram and no abnormal findings could be detected by physical examination of the chest. The leukocyte count was 12,200 per cu. mm.; temperature was 99.2°F. The erythrocyte sedimentation rate was 16 mm. On February 24, 1955, slight increased markings were noted in the right upper lobe without signs of activity. The left lung was normal. The leukocyte count was 7,200 per cu. mm., polymorphonuclear leukocytes, 58 per cent; eosinophils, 2 per cent; lymphocytes, 40 per cent. The sputum was negative for *M. tuberculosis* and *N. asteroides* by smears and cultures. The urinalysis was normal. The temperature was 98.2°F.; pulse, 76 per minute; and respirations, 20 per minute. On a return visit by the patient on May 15, 1955, there was no evidence of active disease, but follow-up observations will continue.

Comment: Examination of the original case report of this patient showed four cultures negative for *M. tuberculosis*. *Nocardia asteroides* was isolated on six occasions by culture and animal inoculation. The marked chronicity of this infection and the facts that resolution did not occur with the usual antimicrobial

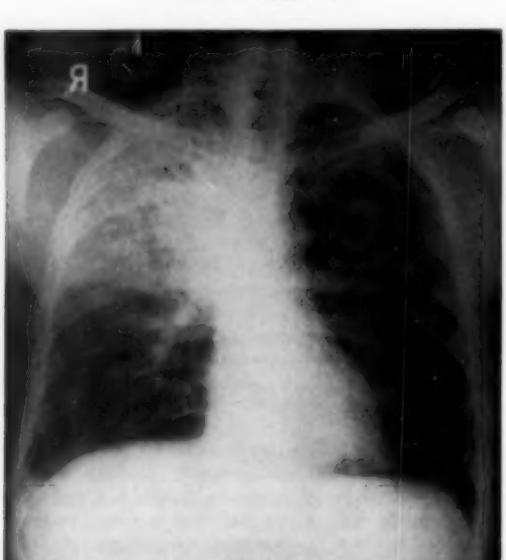

FIG. 4. *Case 3.* Roentgenogram of chest, taken on December 14, 1953, showing an infiltration of the right upper lobe suggestive of lobar pneumonia or tuberculous pneumonia.

drugs, that improvement and clearing of the lesions occurred, and that *Nocardia asteroides*, treated with sulfadiazine, disappeared would lend credence to the diagnosis of pulmonary nocardiosis. As always in such cases, it cannot be established unequivocally that the nocardia were not merely an incidental finding in the sputum and that a bacterial process was present.

Cases in Which the Diagnosis Seems Secure

Case 4 (May 25, 1954). H. W., a 44-year-old married white male farmer and well driller had apparently been in good health until ten days previously when he began running a low-grade fever with shaking chills on several occasions. He stated that he had been sweating slightly at night but had had no frank night sweats. There had been no chest pain, chronic cough, hemoptysis, exertional dyspnea, or swelling of the ankles. The appetite had been very poor for the past three weeks and he claimed that he had lost five pounds. The past and family histories were irrelevant.

Physical examination revealed the patient to be well developed and well nourished. The chest was symmetrical, the trachea was not deviated, and expansion was normal. Many sibilant rales were heard in both right and upper lobes of the lungs. The breath sounds seemed to be suppressed over the right upper lobe. The hemoglobin was 13.4 gm.; leukocyte count, 28,200 per cu. mm.; polymorphonuclear leukocytes, 72 per cent; eosinophils, 1 per cent; lymphocytes, 27 per cent; erythrocyte sedimentation rate, 28 mm.; blood culture was sterile. A series of twelve sputum specimens was negative for acid-fast organisms by the Ziehl-Neelsen stain. A sputum culture was negative for *M. tuberculosis*. A culture on Sabouraud's medium was positive for *N. asteroides*. A roentgenogram of the chest on May 26,

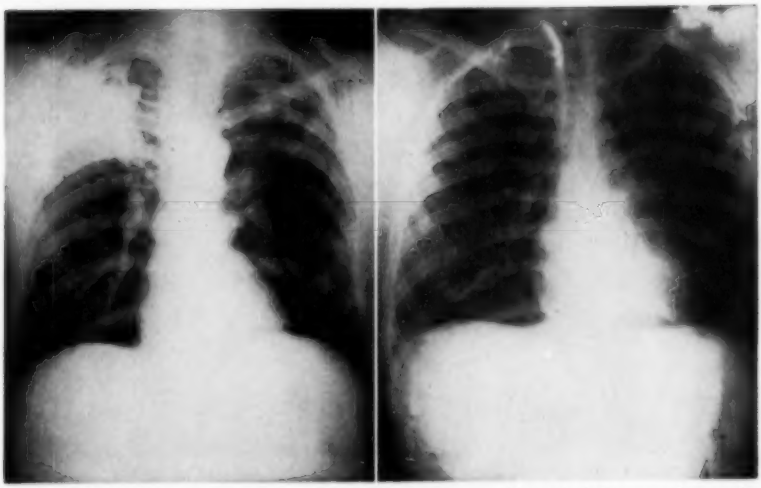

FIG. 5 (Left). *Case 4*. Roentgenogram of chest, taken on May 25, 1954, revealing a dense infiltration with many honeycombed cavities in both upper lobes.

FIG. 6 (Right). *Case 4*. Roentgenogram of chest, taken on October 4, 1954, showing marked clearing of the infiltration in the upper lobes.

1954, revealed the heart to be normal in size and shape. Practically the entire right upper lobe of the lung was involved by a rather dense infiltration, being most dense in the posterosuperior segment. Many honeycombed cavities were noted. The roentgenographic diagnosis was probable tuberculosis in both upper lobes (figure 5). The patient was treated with bed rest, penicillin, and sulfadiazine, 1.0 gm. every four hours. On June 5, 1954, a chest film showed a density in the right apex extending from the level of the clavicle into the third interspace. On the left side, some strandlike infiltration was also noted throughout the upper third. On July 30, 1954, there was evidence of questionable activity on the left side, and questionable activity on the right, resembling a fibroid tuberculosis. By October 4, 1954, the chest roentgenogram showed slight clearing in the apices (figure 6).

On February 17, 1955, no further roentgenographic activity was evident, but fibrosis persisted. The temperature was 98.0°F.; erythrocyte sedimentation rate was 12 mm.; leukocyte count, 8,600 per cu. mm.; differential counts, normal; urinalysis, normal. Cultures for *N. asteroides* and *M. tuberculosis* were negative. Tuberculin and histoplasmin tests were negative. The sulfadiazine was discontinued at this point.

Comment: This patient had six negative cultures for tuberculosis and six isolations of *Nocardia asteroides* by culture and animal inoculation. Likewise, the tuberculin and histoplasmin tests were negative. This was a very chronic pulmonary condition resembling tuberculosis, but without any proof of any tuberculosis and with ample evidence of infection with *Nocardia asteroides*.

Case 5 (December 4, 1950). The patient, A. C., was a 35-year-old white married female spinner who had had a slight cough with expectoration of small amounts of clear mucoid sputum for eight or ten months. No dyspnea, chest pain, fever, or hemoptysis were observed. There had been no recent weight loss; the average weight was 100 pounds. There had been no tuberculosis contacts. Review of the systems was nonrevealing. The family history was negative. The patient states that a "spot" on the left lung had been found in

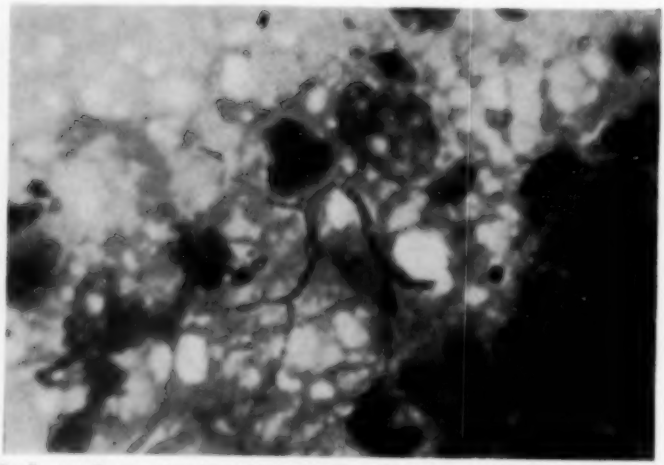

FIG. 7. *Case 5.* Smear showing *Nocardia asteroides* (Gram and Ziehl-Neelsen stains).

1949, and that bed rest for six months was prescribed. A roentgenogram of the chest was said to be negative at the end of that time.

Physical examination revealed a well-developed, slender young woman in no apparent distress. Weight was 101 pounds; temperature was 99.2°F.; pulse, 86 per minute; and respirations, 20 per minute. The trachea was in the mid-line. The thorax was symmetrical, showed normal expansion, and there was normal resonance. Breath sounds were normal throughout the lungs. A few moist rales were audible in the left apex. The hemoglobin was 74 per cent or 12.6 gm.; erythrocyte count, 3,920,000 per. cu. mm.; leukocyte count, 24,200 per cu. mm.; differential counts, 67 per cent polymorphonuclear leukocytes. A blood culture was sterile. Eight sputum specimens were negative for acid-fast organisms. The erythrocyte sedimentation rate was 12 mm. A sputum culture was negative for *M. tuberculosis*. The patient's skin failed to react to histoplasmin, tuberculin (OT 1:1,000), and Brucellergin®. A chest film showed an infiltration in both apices, probably active tuberculosis in the left. The patient left town for a rest in the Western states. Upon return on December 4, 1952, a chest roentgenogram showed no essential changes. Eight sputum specimens were negative for acid-fast organisms; erythrocyte sedimentation rate was 22 mm.; leukocyte count was 9,600 per cu. mm.

The patient was next seen on August 12, 1953, because she had been running a fever for two weeks, had been awakened in the night because of coughing and choking with four or five mouthfuls of blood. Temperature was 98.6°F.; pulse, 88 per minute; respirations, 20 per minute. Her weight was 106 pounds. A roentgenogram of the chest revealed bilateral apical shadows with questionable activity on the left. Physical examination revealed coarse rales in both apices. The hemoglobin was 12.2 gm.; leukocyte count, 10,400 per cu. mm.; differential counts were not remarkable; and the erythrocyte sedimentation rate was 24 mm. A blood culture was sterile and urinalysis was negative. A series of six sputum specimens showed two with faint acid-fast organisms atypical of tubercle bacilli. Gastric washings were negative for tubercle bacilli on microscopy and culture. A sputum culture on blood glucose agar showed *Nocardia asteroides* (figure 7). A tuberculin test (OT 1:1,000) and a histoplasmin test were negative.

The patient was placed on sulfadiazine, 1.0 gm. four times daily, bed rest, and supportive measures for seven months. On August 17, 1954, a roentgenogram of the chest showed the heart and diaphragms to be normal. There were strandlike infiltrations in the apices suggesting fibrosis. Sputum cultures for tubercle bacilli and fungi were negative. The

leukocyte count was 6,700 per cu. mm.; hemoglobin, 13.2 gm.; differential counts, normal; and the erythrocyte sedimentation rate was 8 mm. Temperature was 98.0°F.; weight, 108 pounds. Physical examination showed no abnormalities. The lungs were clear.

A chest roentgenogram on February 25, 1955, showed no signs of activity, but fibrosis persisted in the apices. The other laboratory studies were negative at this time.

Comment: The diagnosis of pulmonary nocardiosis in this case was made by the isolation of *Nocardia asteroides* by culture and animal inoculations on four occasions. The chest roentgenograms suggested pulmonary tuberculosis but cultures for *M. tuberculosis* were negative on six occasions. Likewise, repeated tuberculin tests were negative. In addition, it is believed that the clearing of the infection with sulfadiazine constitutes added evidence of the diagnosis.

Case 6 (September 8, 1951). The patient was a 35-year-old white married saleswoman who had been well until one month previously, when she had begun to have chilly sensations, afternoon fever of one degree, night sweats, aching all over, loss of appetite, and fatigue. Two weeks prior to admission she had begun to cough and had developed a sore throat. Two days later, she spat up a plug that looked yellow and slimy. When first seen, she complained of dyspnea, palpitation, and nervousness. There had been some weight loss. Review of systems was negative. Past history revealed that the patient had had pleurisy fifteen years previously; bronchopneumonia, fourteen years previously; appendectomy, fifteen years previously; and a tonsillectomy in childhood. A roentgenogram of the chest was said to have been normal in May, 1950. The family history was nonrevealing.

The physical examination revealed a young woman with a slight cough, who seemed very anxious. Temperature was 99.8°F.; pulse, 88 per minute; respirations, 22 per minute. Weight was 122 pounds. The throat was injected. The lungs expanded equally. There was slight dullness to percussion over the right apex, in which a few moist rales were present. The packed cell volume was 42 per cent; hemoglobin, 13.2 gm.; the leukocyte count was 16,250 per cu. mm. with 85 per cent segmented, 4 per cent unsegmented, 11 per cent lymphocytes. The erythrocyte sedimentation rate was 22 mm. Blood culture was sterile and urinalysis was normal. A sputum examination revealed small yellow granules which showed mycelia which stained acid-fast by Ziehl-Neelson technique. A culture on blood glucose agar revealed *Nocardia asteroides.* Culture for *M. tuberculosis* was negative. A tuberculin test (OT 1:1,000) and a histoplasmin test were negative.

A chest roentgenogram showed a small area of infiltration in the right apex and a small calcified nodule in the left middle third. The left dome of the diaphragm was flattened. The patient was treated with rest, sulfadiazine (2 gm. initially, then 1 gm. every four hours) until October 18, 1951, when a chest plate revealed only slight haziness in the right apex. The erythrocyte sedimentation rate was 12 mm.; leukocyte count, 12,200 per cu. mm.; differential spread, normal; and urinalysis, negative. The temperature had become normal and she felt much better. Cultures of the sputum were still positive for *Nocardia asteroides.* The sulfadizine was continued, 1.0 gm. four times daily, until February 23, 1952, when a chest film showed no evidence of infiltration in the right apex. The erythrocyte sedimentation rate was then 6 mm.; leukocyte count, 7,200 per cu. mm.; polymorphonuclear leukocytes, 85 per cent; hemoglobin, 12.8 gm.; sputum culture, negative for *N. asteroides;* and the urinalysis, negative.

A chest film on May 20, 1955, revealed the heart, lungs, and diaphragms to be normal. Physical examination was normal; temperature, 98.6°F.; weight, 132 pounds; erythrocyte sedimentation rate, 7 mm.; leukocyte count, 6,850 per cu. mm.; and sputum, negative for nocardia by smear and culture.

Comment: This patient had serial chest roentgenograms suggestive of pulmonary tuberculosis, but with numerous negative smears and four negative cultures for *M. tuberculosis.* Repeated tuberculin tests were negative. There were six

positive cultures for *Nocardia asteroides*. Yellow-orange granules typical of *Nocardia asteroides* were expectorated in the sputum. This exemplifies chronic pulmonary infection which cleared with sulfadiazine and which is typical of pulmonary nocardiosis.

Case with Classical Onset

Case 7. Pulmonary nocardiosis (May 10, 1955). The patient was a 63-year-old white female merchant who was well until approximately sixty days prior to her first visit, at which time she had begun to experience anorexia, fatigue, night sweats, generalized joint aching, and slight dry hacking cough. A small sore on the right hand following penetration of a rose thorn had persisted for two or three weeks, but was never treated. Two weeks prior to her first visit she had developed an irregular fever in the afternoon, had ached in the right shoulder, and had coughed more frequently. One week prior to being seen, she had had about six chills, and had perspired very freely. She had been rather dyspneic and her heart "pounded." The sputum was scant and yellow in appearance. Review of the systems was negative. Past history was not significant. A chest film in 1952 had been considered to be normal. The family history was negative for familial diseases, including tuberculosis.

Upon admission to the hospital the patient was a well-preserved woman with a temperature of 102°F.; pulse, 110 per minute; respirations, 28 per minute. Her skin was hot, moist, and somewhat cyanotic. The neck showed no masses and the trachea was felt in the midline. Expansion was reduced over the right thorax. Percussion seemed resonant except for slight dullness near the right hilar area where bronchial breath sounds and a few scattered coarse rales were audible. The heart was normal in size, rhythm was normal, and no murmurs were heard. Blood pressure was 124 systolic, 76 diastolic in mm. of mercury. There was a small, recently healed scar on the right forefinger. The remainder of the physical examination was normal.

Hemoglobin was 11.2 gm.; leukocyte count was 25,200 per cu. mm., with 78 per cent polymorphonuclear leukocytes. The erythrocyte sedimentation rate was 32 mm.; and urinalysis, negative. Blood and urine cultures were sterile. A sputum specimen, obtained by postural drainage, was greenish yellow mucoid, and revealed gram-positive cocci and bacilli on smear. Cultures revealed *Staphylococcus aureus* and a hemolytic streptococci. A culture of the sputum for *M. tuberculosis* was negative at six weeks. A fungus culture revealed gram-positive acid-fast *Nocardia asteroides*.

The patient's stain failed to react to PPD, 0.00002 mg., and histoplasmin. An electrocardiograph showed findings suggestive of an anterior myocardial wall insufficiency. A chest roentgenogram revealed the heart and diaphragms to be normal, but showed an unresolved consolidation of the right middle lobe of the lung.

Treatment with tetracycline, penicillin, and sulfadiazine produced a fall of the temperature to normal by lysis in five days. On May 15, 1955, a chest film showed less consolidation. On June 4, 1955, a roentgenogram of the chest revealed considerable clearing of the consolidation. Sulfadiazine was continued. On June 15, 1955, a chest film showed patchy infiltration of the right middle lobe. The leukocyte count was 14,600 per cu. mm. The erythrocyte sedimentation rate was 24 mm. Treatment is being continued.

Comment: The illness of this patient started as a rose-thorn wound of the right hand, which healed without treatment. When the pneumonia developed, seven sputum cultures were positive for *Nocardia asteroides* and two sputum cultures were negative for *M. tuberculosis*. Repeated tuberculin tests have been negative. Blood cultures were repeatedly sterile. It is assumed that this infection with nocardia began with the rose-thorn injury of the hand, a type of onset which would be more classical for nocardiosis.

DISCUSSION

The recognition of pulmonary mycoses taxes the ability of the chest physician. Nevertheless, increasing numbers of this group of diseases are being reported and fungal diseases should be considered in any obscure disease of the lungs.

Although only forty-four cases of pulmonary nocardiosis could be collected from the literature, it is probable that many cases pass undetected. The diagnosis of this entity is based upon laboratory diagnosis through identification of the etiologic organism by appropriate smears and cultures. It is fortunate that the nocardia grow rather well on ordinary media under aerobic conditions. As the chief pathogenic species, *Nocardia asteroides*, is acid-fast by Ziehl-Neelson technique, special care is required in differentiating it from *Mycobacterium tuberculosis*. Moreover, as nocardia do not survive the concentrating techniques commonly used in culturing *M. tuberculosis*, cultures for nocardia should be planted prior to concentrating the sputum specimen to culture for tubercle bacilli.

Pulmonary nocardiosis begins as an acute pneumonitis which may become chronic and resemble pulmonary tuberculosis. As a consequence, in any case suggestive of pulmonary tuberculosis in which bacteriologic confirmation is lacking, nocardiosis should be suspected. The differential diagnosis between tuberculosis and nocardiosis is very important because the treatment is very different. Three of the patients in the present series had chest roentgenograms suggestive of apical tuberculosis (*Cases 2*, *5*, and *6*). One patient (*Case 4*) had roentgenographic evidence of honeycombed cavities in the upper lobes, but they cleared following sulfonamides. Another patient (*Case 3*) had chest films suggestive of a pneumococcal or a tuberculous pneumonia of the right upper lobe. The cutaneous reactions to tuberculin and to histoplasmin were negative in all 7 cases. Cultures for *M. tuberculosis* were likewise negative in all cases.

One patient (*Case 1*) had an abscess of the left lower lobe. This patient and one other (*Case 5*) were the only ones who had hemoptyses. Consolidation of the right middle lobe similar to that in reported cases of right middle lobe syndrome was present in *Case 7*.

The differential diagnosis between pulmonary actinomycosis caused by the anaerobic endogenous *Actinomyces bovis*, which is not acid-fast, and pulmonary nocardiosis produced chiefly by the exogenous aerobic acid-fast *N. asteroides* is important. Actinomycosis may respond well to penicillin and other antimicrobials, whereas nocardiosis responds to sulfonamides. In only one of the present cases were yellow-orange granules expectorated (*Case 6*), whereas typical sulfur granules are usually expectorated in actinomycosis sooner or later.

In all 7 cases of the present series, the sputum contained *N. asteroides*. The erythrocyte sedimentation rates were 16 to 32 mm. During the acute illnesses, the leukocyte counts ranged from 10,250 to 26,000 per cu. mm. with a corresponding neutrophilia during the active disease. These findings approximated normal as treatment was pursued. The sputum remains positive for *N. asteroides* in *Cases 1* and *7*.

No correlation was found between the patient's occupation and the occurrence

of nocardiosis in the present series. The group was composed of one farmer and well digger, one housewife, one student, one spinner, one merchant, one warehouse foreman, and one saleswoman.

Treatment with sulfadiazine and symptomatic measures has produced probable cures in 4 cases. Similar treatment is being continued in the 3 other cases. All 7 patients will be followed over a prolonged period for any possible reactivation. It is important to continue the sulfadiazine therapy for many months. The usual precautions in detecting sulfonamide intoxication and blockage of the renal tubules should be carefully maintained.

SUMMARY

Seven cases of pulmonary nocardiosis, substantiated by the isolation and culture of the acid-fast fungus *Nocardia asteroides*, are reported.

Three of these cases simulated apical tuberculosis; one, a nontuberculous bacterial pneumonia of the right upper lobe; and another, a lobar pneumonia of the right middle lobe. An abscess of the left lower lobe was present in another patient; and, in the remaining patient, multiple honeycombed cavities of both upper lobes were present.

Four of the patients are presumed to have been cured following sulfadiazine therapy. The remaining 3 are continuing to receive treatment.

Only forty-four cases of pulmonary involvement with nocardia have been found on review of the literature.

SUMARIO

La Nocardiosis Pulmonar, Repaso con Presentación de Siete Casos

Preséntanse 7 casos de nocardiosis pulmonar, comprobados por el aislamiento y cultivo del hongo ácidorresistente *Nocardia asteroides*.

Tres de estos casos simulaban tuberculosis del vértice, uno neumonía bacteriana no tuberculosa del lóbulo superior derecho y otro neumonía lobular del lóbulo medio derecho. En otro enfermo, hacía un absceso del lóbulo inferior izquierdo y en el enfermo restante, existían cavernas apanaladas múltiples en embos lóbulos superiores.

Cuatro de los enfermos se supone que curaron a continuación de la sulfadiacinoterapia. Los otros 3 continúan recibiendo tratamiento.

En un repaso de la literatura no se han encontrado más que 44 casos de invasión pulmonar por nocardias.

RESUME

Nocardose pulmonaire. Revue de la littérature et rapport sur sept cas

L'auteur fournit un rapport sur sept cas de nocardose pulmonaire confirmée par l'isolement et la culture du champignon acido-résistant, *Nocardia asteroides*.

Parmi ces cas, trois simulaient la tuberculose apicale, un autre la pneumonie bactérienne non tuberculeuse du lobe supérieur droit, un autre la pneumonie lobaire du lobe moyen droit. Il y avait un abcès du lobe inférieur gauche chez un autre malade et, chez le dernier, des lésions cavitaires «en nid d'abeilles» étaient présentes dans les deux lobes supérieurs droits.

Quatre de ces malades sont considérés comme guéris à la suite d'un traitement par la sulfadiazine. Les trois cas restants sont encore en traitement.

Une revue de la littérature n'a révélé que 44 cas de lésions du poumon par nocardia.

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COCCIDIOIDOMYCOSIS[1]

A Review and Presentation of 100 Consecutively Hospitalized Patients

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INTRODUCTION

Coccidioidomycosis, which was known up to a decade ago mainly to physicians and mycologists working in the endemic area, must now be seriously considered in the differential diagnosis of chronic pulmonary lesions in nonendemic areas. Because of the great numbers of military personnel who were stationed in endemic areas during and after World War II, a widespread scattering of the disease occurred, although the area itself apparently showed no signs of expanding.

HISTORY

Wernicke (1) described the first case of coccidioidomycosis in the Chaco region of Argentina in 1892. Four years later Rixford and Gilchrist (2) had discovered 2 cases on the North American continent.

Isolation of the fungus from a patient was first achieved in 1900 by Ophuls and Moffit (3). Thirty-four more years elapsed, however, before it was isolated from the soil of Kern County, California, by Stewart and Meyer (4).

The clinical picture was completed in 1936 by Dickson (5) who correlated "valley fever" or "desert fever" as the primary stage, describing the granuloma or disseminated form as a later, serious manifestation.

Most of our present knowledge regarding skin testing and serology has been contributed by C. E. Smith, who continued Dickson's work.

ENDEMIC AREAS

Outside the United States, the known endemic areas are Argentina, Venezuela, Paraguay, and northern Mexico. There is some question whether Italy should be included.

In the United States the area extends through western Texas, along the Mexican border, through the southern parts of New Mexico, Arizona, and Utah, the tip of Nevada, and then into the highly endemic San Joaquin Valley of California. North of Fresno the disease becomes spotty. Occasionally it spills over the coast range westerly, being found in dry parts of Ventura and Santa Barbara counties. Other spotty areas are found in San Bernardino, Riverside, San Diego, and the Imperial Valley (6).

Sporadic infections have been reported in patients who were never in an endemic area. Two cases were reported in Italy by Jacono in 1930, and a third by

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TABLE 1

100 CASES OF COCCIDIOIDOMYCOSIS

Diagnosis

† Documented	87
(*a*) Resected pulmonary tissue	62
(*b*) Sputums, gastric aspirates, bronchial washings, or extrapulmonary lesions	25
†† Presumptive	13
(*a*) Area, skin and serologic tests positive	7
(*b*) Area, coccodioidin-positive, tuberculin-negative	5
(*c*) *Area, coccidioidin-positive, tuberculin-positive	1

* (Recovery of fungus four years previously with same roentgenographic lesion)

Radaelli and Ciferri in 1934 (7). Montessori (8) discovered 3 patients with the disease in Naples in 1947, one of whom worked with imported grain.

By 1935, 2 patients with coccidioidomycosis had been reported from Hawaii (9). One of them had handled California fruits. Another, who had handled California fruit in Nova Scotia, Canada, developed disseminated disease with fatal termination, described in 1950 by McLetchie and associates (10). Bennett reported a patient in Chicago in 1954 who handled sissal (hemp imported from California and Mexico) while working in a mattress factory (11).

It appears from the above that transmission by fomites occurs, but the incidence is apparently extremely rare.

OBSERVATIONS

The present report consists of an analysis of 100 patients with coccidioidomycosis who were hospitalized consecutively at Fitzsimons Army Hospital from October, 1948, to March, 1955 (table 1).

It must be emphasized that a number of these patients had been away from endemic areas for many years and had long-standing lesions at the time of transfer to Fitzsimons. In some patients, a diagnosis of coccidioidomycosis was documented or presumed prior to transfer (sometimes for excisional surgery) while, in others, either no diagnosis or an incorrect diagnosis of tuberculosis or other disease had been made. Consequently, despite the fact that they represent a consecutive series for a given period, they comprise, to a certain extent, a selective series by reason of transfer and age of disease. Thus, it may be possible that the frequency of a certain symptom or sign may not necessarily be representative of the disease generally, although the percentages quoted follow closely other series published.

GENERAL CHARACTERISTICS OF GROUP

There was naturally a predominance of males, 87 as opposed to only 13 females, because of the nature of the hospital. The age group was from eighteen to fifty-two years, with 64 patients in the twenties. There were 11 Negroes in the group. Three of a total of 4 cases of fatally terminating disseminated disease

occurred in Negroes. The incidence of dissemination of coccidioidomycosis in Negroes is said to be fourteen times that of whites, while their death rate is twenty-three times as great (12).

Ninety-seven of the patients in this series gave a history of having been in at least one endemic area, 39 having been in more than one. Fifty-nine patients had been in California, 41 in Texas, 28 in Arizona, and 7 in New Mexico. The geographical data recorded from 3 patients were too inadequate to ascertain whether or not they had been in any of the above areas.

SYMPTOMS

According to C. E. Smith, 10 to 15 per cent of persons residing in an endemic area for one year and 80 to 90 per cent residing in such an area for ten years will acquire infection. In 60 to 75 per cent this is subclinical and proved only by the development of skin sensitivity. Symptoms, when present, appear after an incubation period of eight to twenty-eight (usually ten to sixteen) days, and vary from a mild upper respiratory infection to a more severe pneumonia-like form.

In the present series, prior to or at the time of entering this hospital, 83 patients were symptomatic. Cough was reported by 70 patients. The cough was generally nonproductive or only slightly productive This is accounted for by the gelatinous consistency of the exudate. Chest pain occurred in 40 patients. It varied from a substernal pressure or unilateral ache to sharp, pleuritic pain. Hemoptysis or streaking was described by 18 patients. Of these, 17 had cavitary disease, the other showing nodular pulmonary infiltrations. Weight loss was noted by 8. Other less frequent symptoms were malaise, fatigue, fever, headache, myalgia, dyspnea, and wheezing (table 2).

PHYSICAL SIGNS

Physical signs were present in 28 patients, of whom 6 had extrapulmonary signs only. Included in the 22 with chest signs were 3 with hydropneumothorax

TABLE 2

100 CASES OF COCCIDIOIDOMYCOSIS

Symptoms	83
Cough	70
Chest pain	40
Bleeding	18
Weight loss	8
Others	
Malaise, Fever, Headache, Dyspnea, Wheezing, Myalgia	less frequently

TABLE 3

100 Cases of Coccidioidomycosis

Signs	28
Pulmonary	22
Parenchymal	17
Pleural	5
Extrapulmonary	6

TABLE 4

100 Cases of Coccidioidomycosis
Predominant Roentgenographic Patterns

Cavitary (single and multiple)	56
Nodular (single and multiple)	34
Infiltrative	8
Pneumonic	8
Hilar mediastinal lymphadenopathy	7
Miliary	4
Hydropneumothorax	3
Pleuritis with effusion	2
Bronchiectasis	2
Atelectasis (middle lobe syndrome)	1

and 2 with pleural effusion, so that only 17 had signs referable to parenchymal pulmonary lesions (table 3).

Erythema nodosum had been a feature in 2 patients and had developed in another during his hospitalization. Usually it appears within the first week of illness and is found in 25 per cent of females as opposed to 5 per cent of males. It is noted less commonly in Negroes than in whites, possibly because of masking by pigmentation. On the other hand, dissemination rarely follows primary infection accompanied by erythema nodosum. Less common manifestations of hypersensitivity are erythema multiforme, varied skin rashes, conjunctivitis, and arthralgia.

Other systemic diseases in the present series were: diabetes mellitus, three; panhypopituitarism, one; and epilepsy, one. There was nothing unusual about the behavior of the coccidioidomycosis in these cases to warrant comment.

ROENTGENOGRAMS

Roentgenographic abnormalities in the chest film are usually found in 80 per cent of the patients with coccidioidomycosis who require hospitalization. The abnormalities generally clear in one to eight weeks, but may persist for two months in a minority. The persisting abnormalities may eventually clear or stabilize as residual lesions but, in approximately 10 per cent of the patients with such residual lesions, the disease disseminates (13).

In the present series, upper lobe lesions were found in 67 cases in contrast to lower lobe lesions in 20 (table 4). The remaining 13 cases represented all lobes

or combinations of lobes. The lesions were right-sided in 58, left in 36, and bilateral in 6 patients.

Cavity: An attempt was made to determine how frequently the classical thin-walled cavity was found in contrast to a thick-walled type. Since the adjectives "thin" and "thick" are somewhat vague, depending on subjective impression, the cavity walls were measured on the roentgenograms and 3 mm. was regarded as the dividing line. Where there was a variation in thickness, an average was taken. Using these criteria, 33 of the cavities were thin-walled and 26 were classified as thick-walled.

Much interchangeability was noted between nodules and both types of cavities. In a typical sequence, the center of a nodule sloughed and formed a small, round, thick-walled cavity. Tension would then develop and the cavity would become larger and thin-walled. In some instances, the cavitary lesion would then regress and leave a nodular residual.

Some writers have described a different pathologic mechanism for the formation of the two types of cavity; but the present writers believe that they are essentially the same and represent different stages of the same process.

Nodules: "Coin lesions" with central radiolucency were considered cavities, and lesions without the central radiolucency were designated as nodules. All of the excised nodules had soft, necrotic centers pathologically, and sometimes bronchial communication was demonstrated. Tiny satellites adjacent to cavities were not included in the categorization of nodules.

Pneumonia and infiltration: Pneumonic lesions and infiltrates may be classified together, since frequently an infiltration represents the residual of a pneumonic lesion. Pneumonitis may take as long as two years to resolve. In the present series the pneumonic densities varied from small hilar foci to lesions which occupied more than one-third of a lung.

Hilar and mediastinal lymphadenopathy: In a series of 75 cases of primary disease observed by Colburn (14), hilar lymphadenopathy alone was noted in 24 per cent; whereas, in another 39 per cent it was seen in conjunction with bronchopneumonia. It is seen less commonly as a residual lesion, a fact borne out by this series.

Pleural effusion: In one patient, a pleural effusion extended up to the scapula while, in another, the fluid obscured the costophrenic angle. In Sweigert's series (15) pleural effusion was seen in 2.6 per cent of the cases. At surgery or autopsy, evidence is found which indicates that small, fibrinous pleural effusions are more common. Rarely does massive effusion occur.

Bronchiectasis and atelectasis: There were 2 patients with bronchiectasis in the present study and one with atelectasis which occurred as a middle lobe syndrome due to hilar adenopathy.

ENDOGENOUS REINFECTION

Endogenous reinfection in coccidioidomycosis as described by Forbus (16) may come from a lesion outside the lung and adjacent tissues or it may develop from a destructive lesion in a bronchus, perhaps in association with adjacent

necrotic, infected lymph nodes. A somewhat similar bronchial spread may take place during the course of the primary infection, merely through aspiration of infected bronchial contents from one part of the lung to another.

There were 4 such patients in the present series, all with cavitary lesions and positive mycology. Local "spread" about the cavity was seen in 3, while in the fourth a new lesion appeared in the contralateral lung following bronchoscopy.

SKIN REACTION TO COCCIDIOIDIN

In order to obtain accurate results in skin testing, the following possible errors should be kept in mind:

(*1*) The use of glassware in which other biologicals have been adsorbed. It should be remembered that autoclaving does not inactivate coccidioidin. Syringes should either be soaked overnight or boiled in dichromate solution.

(*2*) Contaminating the diluted coccidioidin.

(*3*) Injecting an insufficient amount or injecting it subcutaneously instead of intradermally.

(*4*) Reading too soon or too late. Twenty-four- and forty-eight-hour readings are necessary (17). Sometimes induration producing only a faint pink coloration is missed.

Coccidioidin is not antigenic; it does not interfere with the serologic tests, nor does it reactivate old, quiescent lesions (18). The test becomes positive in one to four weeks. The degree of reaction then rapidly increases, becoming highest in those in whom there are other signs of hypersensitivity. In such cases, a 1:10,000 dilution should be used to avoid aggravation. Slow decrease in the degree of reaction over a decade or more is frequently observed. A 1:10 dilution should be used in long-standing residual lesions negative to 1:100.

Occasionally cases of long-standing residuals may not react even to a dilution of 1:10. In a series of patients with cavitary lesions and cultures positive for *Coccidioides immitis* recorded by Smith, 16 per cent were negative cutaneous reactors to coccidioidin, 1:100, and 3 per cent were negative to 1:10. When dissemination of the infection is present, more than 70 per cent of the patients will fail to show skin reactivity to coccidioidin, even in a 1:10 dilution.

The test is highly specific except when it is necessary to use undiluted or only slightly diluted coccidioidin. Cross-reactions to histoplasmin, blastomycin, and haplosporangen may then occur (18).

In cross-reactions with histoplasmin, Groover and associates (19) observed that those predominantly sensitive to histoplasmin, i.e., reacting strongly to 1:1,000, usually showed a mild reaction to coccidioidin, 1:10. In contrast, the subjects predominantly sensitive to coccidioidin showed a mild reaction to histoplasmin, 1:10.

The coccidioidin skin test was performed in 96 patients of the present series and was positive in 71 (table 5). The 1:1,000 and 1:100 dilutions were used in most of the tests. Only 12 patients were tested with a 1:10 dilution of antigen, and of these 8 gave positive reactions. Positive reactions to histoplasmin were recorded in 27 of the patients, of whom 3 were nonreactors to coccidioidin. In those cross-reacting to histoplasmin the sensitivity to coccidioidin was greater in all but one. Fourteen patients with negative skin reactions to coccidioidin and histoplasmin showed positive reactions to tuberculin. There were no cases in

TABLE 5

100 CASES OF COCCIDIOIDOMYCOSIS

Skin Tests	Total			Cavitary			Nodular			Disseminated			Others		
	Positive	Negative	Not Reported	Positive	Negative	Not Reported	Positive	Negative	Not Reported	Positive	Negative	Not Reported	Positive	Negative	Not Reported
Coccidioidin	71	25	4	40	15	0	23	5	3	2	3	1	6	2	0
Histoplasmin	28	61	11	17	36	2	8	19	4	0	3	3	3	3	2
Tuberculin	62	35	3	38	17	0	20	10	1	0	1	2	4	4	0
Coccidioidin-histoplasmin	25			14			8			0			3		
Coccidioidin-histoplasmin-tuberculin	19	8		9	4		7	1		0	2		3	1	

which the histoplasmin reaction was positive while both the coccidioidin and tuberculin reactions were negative.

In 40 of the 55 cavitary cases the coccidioidin reaction was positive and in 15 cases the reaction was negative. Nine of the 12 patients tested with 1:10 dilution had cavitary lesions and, of these, 7 gave positive reactions. If a 1:10 dilution of coccidioidin had been used on all who were negative to the 1:100 dilution, it is probable that a higher percentage of positive reactors would have been obtained. Of the 15 patients negative to coccidioidin, 11 were tuberculin-positive. *Coccidioides immitis* was isolated from 3 of these patients; tuberculosis had been strongly suspected in the remaining 8 until its exclusion by tissue examination.

In the 31 nodular cases, the coccidioidin reaction was positive in 23, and in 5 the reaction was negative. The results for 3 patients were not reported.

In 2 of the disseminated cases the skin reaction to coccidioidin was positive; in 3 cases it was negative; and in one case the result was not reported. The non-reported and negative cases were fatal.

There were 6 positive and 2 negative reactors to coccidioidin among the 8 patients not included in the categories described above.

SEROLOGIC TESTS FOR COCCIDIOIDAL INFECTION

Smith states that, if both precipitin and complement-fixation tests are done repeatedly in patients whose disease is so severe as to require hospitalization, the results will be diagnostic in more than 90 per cent. If dissemination develops, more than 99 per cent will yield positive serologic results (18).

In 107 cavitary cases of coccidioidomycosis with positive cultures, Smith found positive complement-fixation reactions in 60 per cent, while in 25 per cent the reaction was entirely negative (18). The serologic tests are rarely positive in patients with asymptomatic pulmonary residuals, but nevertheless the tests should be done. In Smith's series, in no patient with active primary infection without concomitant or impending dissemination were the serologic tests positive in the presence of a negative skin test (20).

Precipitins may be present as early as the first week after the onset of symptoms and are almost always present by the end of the third week. No reaction is elicited after three to four months regardless of the progress of the disease. Hence, the precipitin reaction is diagnostic, but not prognostic. Only a 1:10 dilution of the antigen need be used since the

TABLE 6

100 CASES OF COCCIDIOIDOMYCOSIS

Serologic Tests	Total			Cavitary			Nodular			Disseminated			Others		
	Positive	Negative	Not Reported	Positive	Negative	Not Reported	Positive	Negative	Not Reported	Positive	Negative	Not Reported	Positive	Negative	Not Reported
	27	43	30	15	28	12	2	14	15	3	1	2	7	0	1

titer is not significant. Precipitin reactions have not been noted to reappear, with one exception in a patient in Smith's series with hydropneumothorax (20).

The complement-fixing antibodies appear more slowly. Only 10 per cent of the patients whose serums ultimately fix complement show the antibodies in the first week. Conversions continue into the third or fourth month. The complement-fixation reaction may remain positive for years, even with uncomplicated infection, whereas in disseminated coccidioidomycosis it may persist indefinitely even after recovery. With residual lesions, however, the complement-fixation reaction is most commonly negative or equivocal after six months.

The titer of the complement-fixation reaction shows some correlation with the severity of infection; when the reaction is positive in greater than a 1:16 dilution, it is likely to indicate dissemination. A 4-plus reaction to the 1:2 dilution is considered diagnostic of coccidioidomycosis. Smith states that more than 40 per cent of 1,800 patients with primary disease whose serums fixed complement were positive in a 1:2 dilution or less; only 2.5 per cent of the reactions were positive in a titer higher than 1:16. In 328 cavitary cases with positive complement-fixation reactions, the titer was 1:2 in 50 per cent and in only 1 per cent were the titers more than 1:16. On the other hand, in 60 per cent of 400 disseminated cases the titer of the reaction was more than 1:16, and in 90 per cent of the fatal cases the titer was even higher (18). The prognosis is considered to be excellent when a high skin sensitivity is present with a negative or low-titered complement fixation. When a pulmonary lesion is increasing despite a stable or decreasing serologic titer, a cause other than coccidioidomycosis should be sought (18).

The results of serologic tests were available for 70 cases in the present series. In 27 of the group, one or more tests were positive (table 6). Precipitins were present in 5 patients, 4 with pneumonia and one with disseminated disease.

Complement fixation: The 27 cases with positive complement-fixation reactions were represented as follows:

	Titer
Cavitary	15 (1:2 to 1:100)
Infiltrative	4 (1:4 to 1:40)
Disseminated	3 (1:8 to 1:256)
Nodular	2 (1:4 to 1:60)
Hydropneumothorax	1 (1:20)
Pneumonic	1 (1:40)
Lymphadenopathy (hilar)	1 (1:2)

The titers of the positive complement-fixation reactions ranged from 1:12 to 1:256. The highest, as expected, were found in the patients with fatal disseminated disease.

Serologic cross-reactions with histoplasmin occurred in 11 patients; 7 of these also reacted to blastomycin. Collodion agglutination for histoplasmosis was positive in 8 cases. This concurs with the findings of Campbell and Binkley (21).

MORPHOLOGY AND CULTURAL CHARACTERISTICS OF COCCIDIOIDES IMMITIS

Coccidioides immitis occurs in two phases: a saprophytic form which occurs in nature and is characterized by mycelia, hyphae, and arthrospores; and a parasitic form which occurs in man and animals and is represented by spherules and endospores.

Coccidioidal infection of rodents, dogs, cattle, sheep, swine, monkeys, and a gorilla have been reported. The respiratory tract is the only portal of entry except in some laboratory-incurred cases.

The spherules become rounded with doubly refractile walls, liberating endospores by cleavage of protoplasm. The cycle repeats itself. With death of the host or unfavorable environment, germ tubes push out from the endospores or spherules, forming hyphae and mycelia, from which the arthrospores are formed (21*a*).

It should be added that arthrospores are often called chlamydospores and spherules termed sporangia by those who believe that *Coccidioides immitis* is one of the family of *Phycomycetes* rather than *Fungi imperfecti*. Both saprophytic and parasitic phases can be maintained indefinitely without altering the character of the organism.

The organisms may be grown on a variety of media. To identify the organism in the young stages, a blood medium should be used. After approximately forty hours at 37°C., the colonies become raised and glistening with a purple coloration beneath, which extends from center to edge, but not beyond, when viewed with transmitted light. This is the best stage for identification. After seventy-two hours colonies may become "fluffy" or "cottony" in appearance, with a prickly effect due to hyphae in which the arthrospores will develop. Pigmentation will vary according to the age of the colony, the medium, and the strain. Gray-brown represents the usual color, but shades of pink and orange may appear. In forty-eight to ninety-six hours a few colonies should be transferred to any of the common nutrient broths. This is not only safer than dealing with plates of fluffy colonies, but provides sufficient mycelium without arthrospores to insure a good reaction in the mouse and produce sufficient spherules for confirmation (22).

Rosenthal and Routien (23) found that, by instilling spherule-containing sputum or exudate from human or animal sources into the bronchi of guinea pigs and propelling the material by air pressure into the finer ramifications of bronchioles and alveoli, it was possible to produce the disease in 100 per cent of the animals. The lesions were for the most part in the upper portions of the lungs, being single or multiple, with a lymph node component, rather than generalized. They thus simulated human infections. The experiments of these investigators showed that spherules can be infective through the respiratory route from man to animal and from animal to animal. It was their conclusion that active primary or progressive coccidioidomycosis in human beings should be considered contagious until proved otherwise. It should be mentioned, however, that forceful propelling by mechanical introduction of air may constitute an entirely different mechanism than that found in ordinary inhalation.

Coccidioides immitis was isolated from 41 of the 91 patients of the present series upon whom cultural studies were made. In 35, the organism was isolated from sputum, bronchial washings, or gastric aspirates; in 5 others, from skin lesions; and in one, from thoracentesis of a hydropneumothorax.

The pathologic distribution of the 35 cases was: cavitary in 28, nodular in 4, pneumonic in one, infiltrative in one, and disseminated in one. As may be seen, the cavitary lesions provided the best opportunity for isolation of the fungus.

In the 35 patients, the sputum alone was positive for *Coccidioides immitis* in 13, the bronchial washings alone in 8, and the gastric aspirates alone in 3. From this it may be seen that not only should repeated testing be done, but all of the

TABLE 7

100 CASES OF COCCIDIOIDOMYCOSIS

Culture for *Coccidioides immitis*	Total			Cavitary			Nodular			Disseminated			Others		
	Positive	Negative	Not Reported	Positive	Negative	Not Reported	Positive	Negative	Not Reported	Positive	Negative	Not Reported	Positive	Negative	Not Reported
	41	50	9	28			4			6			3		
Sputums	21			16			3			0			2		
Bronchial washings	19			17			1			1			0		
Gastric aspirates	6			5			0			1			0		
Extrapulmonary										6					
Tissue	63			35			26			0			2		
Tissue culture	42	20	1	30	4	1	11	15	0				1	1	

available means should be used in an effort to isolate the organism for diagnosis (table 7).

TREATMENT

The medical treatment of coccidioidomycosis is singularly ineffective. Varied drugs, including the heavy metals, ethyl vanillate, gentian violet, sodium caprylate, protoanemonin, nystatin, fracidin, thiolutin, stilbamidine, actidione, prodigiosin, and the common antimicrobial drugs, have all been tried without success in disseminated disease.

Resection of residual lesions has gained increased support during the past decade. Winn (6) believes that there is no need to fear spread from cavities. He states that no treatment is indicated in cavities less than 2 cm. in diameter (25 per cent), since they usually close spontaneously. He has used collapse therapy with some success in cavities between 2 and 4 cm., which are not too peripherally located. This therapy has consisted of pneumothorax or pneumoperitoneum, sometimes supplanted by phrenic nerve paralysis. In cavities more than 4 cm., or peripheral in location, Winn prefers surgical resection.

Cotton and Birsner (24) reported on 30 resections in September, 1950. They found collapse therapy of little value, whereas considerable success was attained by surgery. At first they were somewhat fearful of operating on active disease, especially in the face of positive serologic tests. However, no untoward complications resulted in such cases. They are convinced that the complications to be feared are those which occur solely from the mechanical standpoint.

In some of the resected specimens in the Cotton-Birsner series, cavities which had appeared thin-walled were found to have a dense, fibrous wall, difficult to compress manually. In others, dense adhesions were present from the cavity wall to the thoracic cage. It is obvious that collapse therapy could not be effective in such cases. Cotton and Birsner regarded the following as indications for the surgical therapy of coccidioidomycosis:

Special types of cavities such as giant cavities, those secondarily infected, or blocked cavities

A ruptured cavity with spontaneous pneumothorax or empyema

Nonexpansible lung
Continued or severe hemoptysis
An expanding lesion, the so-called coccidioidoma
Failure of medical (including collapse) therapy

Forsee and Perkins (25) published a series of 50 resections, and it was their opinion that this represented the treatment of choice both for cavitary and solid residuals, not only to establish diagnosis, but to prevent complications.

One complication described by several authors (25–27) was the appearance of a new cavity in another lobe after segmental or lobar resection. Whether this was due to overexpansion postoperatively or to the resection of an insufficient amount of tissue is difficult to estimate.

Surgery was performed on 62 patients in the present series. Ten of the patients received medical treatment for four months or more preoperatively, with tuberculosis the presumptive diagnosis in 9; the other patient was treated for war wounds before discovery of his lesion. In those who received early surgery, the diagnosis of coccodioidomycosis had been established or strongly suspected prior to transfer to this hospital.

The resected lesions were cavitary in 34 patients, nodular in 25, and nodular and bronchiectatic in one patient. A hydropneumothorax was present in one and bronchiectasis alone in one patient.

The procedure varied with the type and extent of the disease. A combined lobectomy and segmental resection was done on one large cavitary lesion. Lobectomy alone was performed in 17 patients; segmental and wedge resections in 2; segmental resection alone in 10 patients; a wedge resection in 3 patients; a ligulectomy and decortication in one; and a decortication alone in one patient.

More extensive surgery was performed for cavitary disease, wedge resection being done primarily for nodular lesions. Complications were of a minor nature in all but one patient, who developed a new cavity postoperatively. Serosanguineous effusions developed in 4 and empyema in 3 patients. These all cleared after repeated aspirations. *Coccidioides immitis* was cultured from the three empyemas. Pneumothorax was a feature in 3, the collapsed lung expanding prior to discharge.

PATHOLOGY

From the clinician's standpoint the disease may be classified as pulmonary or disseminated.

The pathology of asymptomatic primary disease is unknown. The changes noted in symptomatic primary disease are based mainly on the interpretation of serial roentgenograms during close follow-up of epidemics. Hilar and mediastinal lymph node enlargement and bronchial inflammatory changes are prominent. Focal or lobar consolidation of the pulmonary parenchyma, sometimes of a distinctly miliary type but more often as a homogenous, diffuse, lobar consolidation, is encountered as well as solitary areas of consolidation, sometimes with sharply defined cavity formation. Pleural effusion occurs and is usually small; occasionally it is massive. Multiple, more or less isolated areas of consolidation with intervening disseminated focal miliary lesions have also been observed (28).

TABLE 8

COCCIDIOIDOMYCOSIS AND TUBERCULOSIS

Patient	Lesions	Skin Tests			Serologic Tests	Culture for *Coccidioides Immitis*	Acid-Fast Bacilli	Surgery	Notes
		Coccidioidin	Histoplasmin	Tuberculin					
1	Nodule LUL	Not reported	Not reported	Not reported	Not reported	Sputum	1 smear and culture, preadmission; 1 gastric aspirate, FAH*	Wedge resection, spherules present, culture negative for *Coccidioides immitis*	Coexisting tuberculosis and coccidioidomycosis?
2	Multiple thin-walled cavities, RUL	+	0	+	Complement-fixation, 1:10	Gastric aspirates and mouse culture	1 gastric aspirate preadmission, 2 gastric aspirates FAH	None	Coexisting tuberculosis and coccidioidomycosis?
3	Thick-walled cavity, LUL	0	0	+	No reaction	2 gastric aspirates	1 gastric aspirate	None	Coexisting tuberculosis and coccidioidomycosis?
4	Infiltration RML; large right pleural effusion	+	+	+	Complement-fixation, 1:32	Negative	Negative	Total right pleurectomy; empyema cavity; positive on smear, stain, and culture for acid-fast bacilli	Coexisting tuberculosis and coccidioidomycosis?
5	Thin-walled cavity RUL	0	0	+	No reaction	Negative	1 sputum smear, 1 gastric aspirate preadmission	None	Cavity present four years regressed in four months to small nodule

6	Thick-walled cavity, RLL	0	0	+	Negative	Negative	1 smear	Lobectomy; spherules present, culture positive for *C. immitis*	Probable false bacteriology
7	Infiltration LUL	+	0	0	Complement-fixation, 1:80	Sputum	1 sputum smear preadmission; 1 sputum smear FAH	None	Probable false bacteriology
8	Infiltration LUL	0	0	+	No reaction	No reaction	2 sputum smears	Wedge resection; spherules and culture positive for *C. immitis*	Probable false bacteriology

* FAH—Fitzsimons Army Hospital

Healed or progressively healing lesions may be separated chiefly into coccidioidoma or the solitary or multiple thin-walled cavities. The coccidioidoma are solitary or multiple lesions 1 to 3 cm. or more in diameter. These are well encapsulated and represent a healed focus of pneumonia. Calcification may be present, but, according to Forbus and others, this is unusual. The solitary or multiple thin-walled cavities are lined by chronic granulation tissue or partially by metaplastic bronchial epithelium. Both Forbus and Smith believe that these residuals represent a potential source of dissemination.

Meningitis is present in 25 per cent of disseminated cases. The over-all mortality is 50 to 60 per cent. Dissemination may result in only a single extrapulmonary focus. Smith observed that in 419 patients with extrapulmonary lesions, 75 (18 per cent) could be so classified.

Very few writers have described the finding of the mycelial form of *Coccidioides immitis* in autopsy material or resected tissue. Forbus noted it in only one of the 95 autopsies of disseminated cases he studied. Puckett (29), by careful study of the entire resected lesions with special staining technique, demonstrated the mycelial forms in 34 of 64 resected specimens. They were present in 25 of 34 cavitary lesions and in 9 of 30 coin lesions. Mycelia and spherules were seldom present in the wall of the same cavity.

Puckett also noted that, in the study of solid granulomatous lesions, the intact spherule with endospores was frequently difficult to find, while fragments of spherules and scattered endospores of various sizes were numerous.

Spherules or endospores were demonstrated in the entire 62 resected specimens of the present series.[3] In addition, mycelia were noted in 34 specimens, 25 of which represented cavities.

Cultures positive for *Coccidioides immitis* were obtained in 41 patients and were confirmed by mouse inoculation in all cases. Of these, 30 were cavitary and 11 were nodular cases.

COEXISTING TUBERCULOSIS AND COCCIDIOIDOMYCOSIS

In table 8 are the data on 8 cases in which some confusion existed because of the isolation of acid-fast bacilli on one or more occasions. In one case, the evidence for the presence of both coccidioidomycosis and tuberculosis is well documented while, in 3 others, strong presumptive evidence is present. In the remaining 4 cases, in which only smears were positive for acid-fast bacilli, it is believed that there was insufficient evidence to support the diagnosis of concomitant tuberculosis.

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Figures 1–11 follow

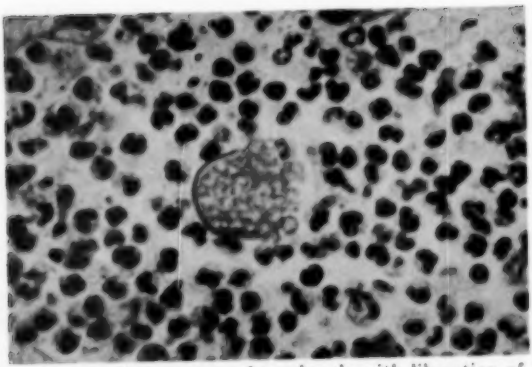

FIG. 1. Microgram showing rupture of a spherule with liberation of endospores.

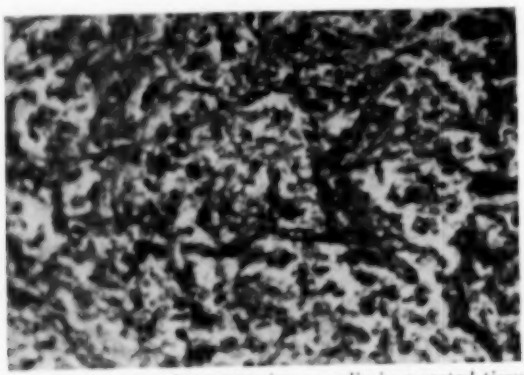

FIG. 2. Microgram demonstrating mycelia in resected tissue.

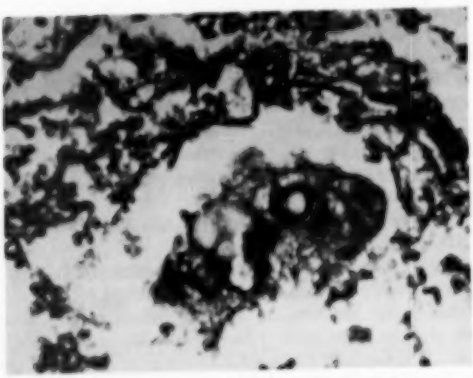

FIG. 3. Microgram demonstrating spherules and mycelia in resected tissue.

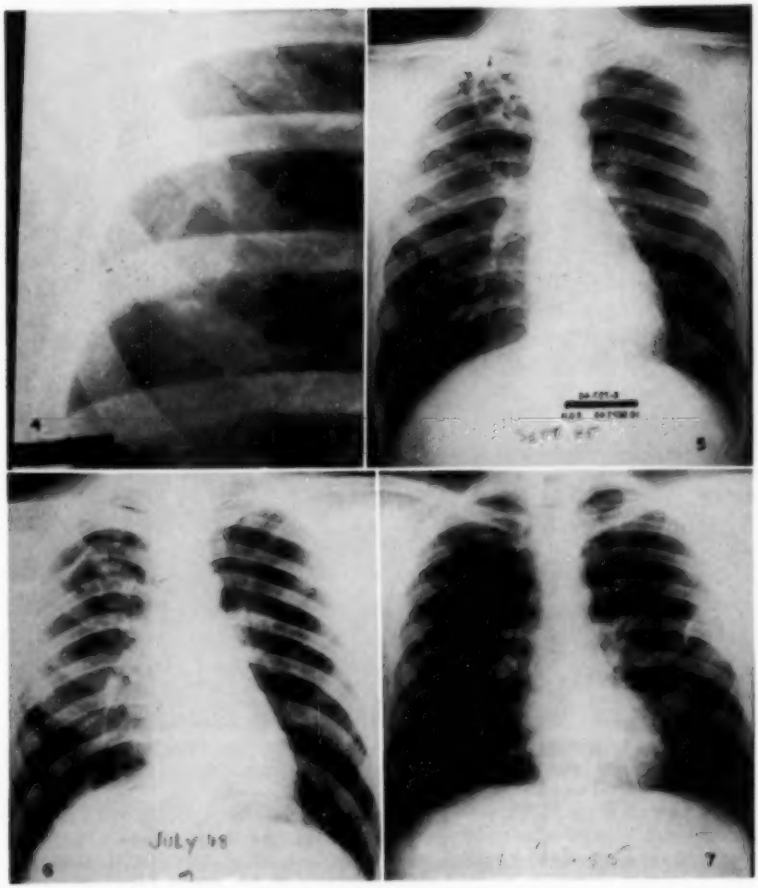

FIG. 4. "Typical thin-walled" cavity.

FIG. 5. Chest roentgenogram showing cavitary lesion due to coccidioidomycosis, right upper lobe. This was removed by lobectomy.

FIG. 6. Chest roentgenogram demonstrating new cavitary lesion due to coccidioidomycosis two and one-half years following lobectomy for right upper lobe cavity. Same patient as figure 5.

FIG. 7. Chest roentgenogram showing cavitary lesion in left midlung field ten days before bronchoscopy.

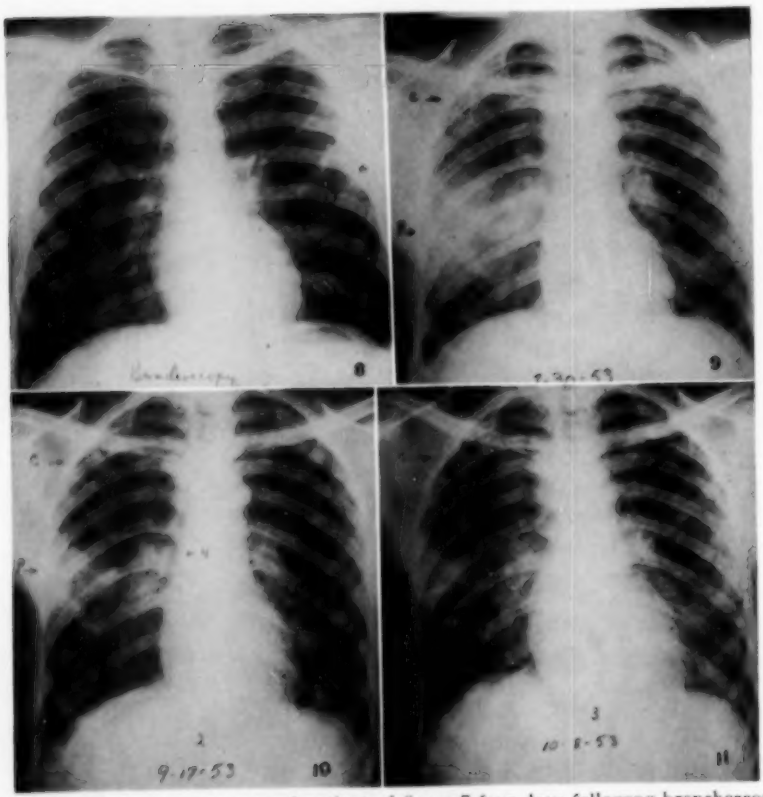

FIG. 8. Chest roentgenogram of patient of figure 7 four days following bronchoscopy. There has been bronchogenic spread to the right. Sputum positive for *C. immitis* was obtained for the first time following demonstration of this spread.

FIG. 9. Chest roentgenogram showing several of the roentgenographic patterns of coccidioidomycosis: enlarged hilar nodes; an area of pneumonia in right midlung field; a thin-walled cavitary lesion in the right upper lobe with small satellite nodules below in the second interspace anteriorly; and a fine nodular pattern throughout both lung fields due to hematogenous dissemination.

FIG. 10. Chest roentgenogram of patient of figure 9 (seven weeks later), showing clearing of the area of pneumonia. Enlarged hilar nodes, cavitary, nodular, and disseminated lesions remain unchanged.

FIG. 11. Chest roentgenogram of patient of figure 9 (three weeks later), showing further clearing of area of pneumonia in right midlung field. The disseminated lesions throughout both lung fields are more apparent. The other lesions remain essentially unchanged. The patient died shortly after this film was obtained.

THE REFLEX RESPONSES TO INFLATION OR DEFLATION OF THE LUNGS AND THEIR IMPORTANCE IN THE REGULATION OF RESPIRATION[1]

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INTRODUCTION

Since the classical description of Hering and Breuer in 1868 (1), it is known that inflation or deflation of the lungs produces marked changes in the rate of breathing. However, very little attention has been given to the possible importance of this knowledge in the regulation of respiratory activity. In all of the reviews on the control of respiration, this control is mainly attributed to oxygen and carbon dioxide tensions and to hydrogen ion concentration (2). The inflation or deflation reflex responses are accorded only a small part in determining the frequency and length of the cycle of the respiratory movements. With the growth of thoracic surgery, however, cases of inflation or collapse of part or all of a lung are seen daily, and it is necessary to consider to what extent the accompanying respiratory changes can be due to the activity of these reflexes.

In some patients in whom part of the lung tissue has been excised, and in whom the resection has not been followed by thoracoplasty, a great reduction in diaphragm movement is often reported. This reduction is commonly ascribed to muscle paralysis, an explanation that is not always satisfactory. In these patients, the remnant lung tissue is stretched in order to fill the chest cavity, and the lung will be in the same condition as an experimentally inflated lung. The same can be said if part of the lung is destroyed by a pathologic process.

There are some other reports of dyspneic patients, particularly with emphysema, in whom the collapse of the distended lung has been followed by a reduction of the dyspnea. The question is: Does the Hering-Breuer reflex maintain inhibited respiratory movements for long periods of time, even if there is some degree of superimposed asphyxia? The purpose of the present study is to try to obtain experimental data to answer that question.

It was believed that, first, a quantitative study of the reflexes was needed; next, an attempt should be made to see if the characteristics of accomodation were such that they could explain the observations after long periods of time; and finally, a comparison should be made of the relative importance of the reflexes upon the control of breathing when induced under conditions of altered oxygen and carbon dioxide tensions.

It is recognized that inflation or deflation of the lungs stimulates different kinds of receptors which can give rise to different types of responses (3). In the present paper, however, as the stimulus intensities were kept within the proper limits, the designation of Hering-Breuer reflexes is used to describe the inhibition

[1] From the Department of Physiology, Instituto Nacional de Neumología, Tlalpan, D. F., México.

of respiration following inflation and the increased breathing activity after deflation of the lungs.

METHOD

Rabbits under pentobarbital sodium (Nembutal®) anesthesia were used. A glass tracheal cannula with three external openings was inserted. To one of them was connected the system which permitted change of intrapulmonary pressure. At another, a thermocouple (constantan-copper) was placed to measure the changes in pulmonary ventilation (the rate of gas flow in and out of the lung was indicated by the rate of cooling or warming of the junction). The reference junction was in a thermostat at room temperature. The third opening was either left exposed to the atmospheric air or could be connected to a spirometer.

The system for intrapulmonary pressure changes consisted of what was essentially a container of approximately one gallon capacity, in which a continuous pressure, either positive or negative, was maintained by means of a pressure or a vacuum pump. By working one of these pumps continuously, and by means of an adjustable leak in the system, it was possible to have a fixed sustained pressure and to reduce to a minimum the transient changes which occur when connecting the system to the lungs. The pressure was read in a mercury manometer.

Complementing the information obtained with the thermocouple, particularly during the time in which the stimuli were being applied or when the tracheal cannula was closed, was the electrical activity of the diaphragm muscle, recorded by means of fine steel serrefine electrodes placed over the anterior surface of the muscle fibers which insert themselves at the xiphoideal appendix. In some cases, the oxygen saturation of the arterial blood was determined by means of an earpiece of the Wood type and a Grass balance-demodulator unit. Routinely, an electrocardiogram was also taken. All of these electrical records were obtained on a three-channel, ink-writing oscillograph (Grass electroencephalograph model III D).

The intrapulmonary pressures applied were made to vary between minus 20 to plus 20 mm. of mercury. The animal could be breathing air all the time, or could be breathing the gas mixtures in the bell of the spirometer. Sometimes, however, during the application of the stimulus, the third opening in the tracheal cannula was closed, thus bringing the animal to conditions of asphyxia. Routinely, a negative pressure was followed by a positive one in order to prevent changes due to hysteresis of the lung tissue.

Ventilation was judged by the amplitude of the records given by the thermocouple and by integrating in time the records of the electrical activity of the diaphragm, as described in an earlier paper from this department (4). In neither case were absolute values considered.

RESULTS

The Stimulus-Response Relationship

The respiratory responses obtained during short periods of inflation or deflation of the lung are proportional to the strength of the stimulus, i.e., to the magnitude of the pressure applied. As is known, they consist of the inhibition of respiration with positive pressures and the increase in respiratory activity with negative pressures (figure 1).

The responses were taken to be the relationship between either ventilatory or electrical activity of the diaphragm, integrated for the first cycle following the stimulus, and the activity for the same period of time during control conditions.

The curves expressing that relationship changed with different experimental conditions. In general, they were of the type shown in figure 2. It may be noted that the threshold could be as low as 1 mm. of mercury.

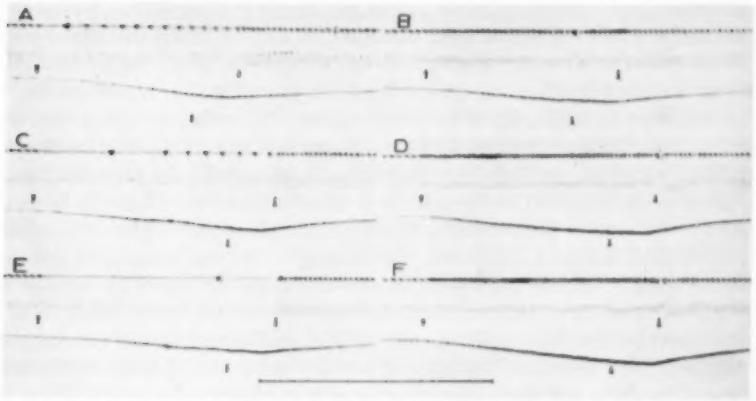

FIG. 1. Typical responses to inflation (A, C, E) and deflation (B, D, F) of the lung. In each instance, the upper record is the electromyogram of the diaphragm; the middle record is from the thermocouple in the tracheal cannula, inspiration upwards; and the lower record is that of the oxygen saturation of arterial blood. Between the arrow marks a pressure was applied of 2, 4, and 8 mm. of mercury for A, C and E, respectively, with the trachea occluded. The same values, but of negative pressure for the corresponding records, are in the same row. The inverted arrow below each oxygen saturation record corresponds in time with the end of the stimuli applied, since the measurement was recorded with a longer pen and is, therefore, displaced to the left. The horizontal line at the bottom represents thirty seconds.

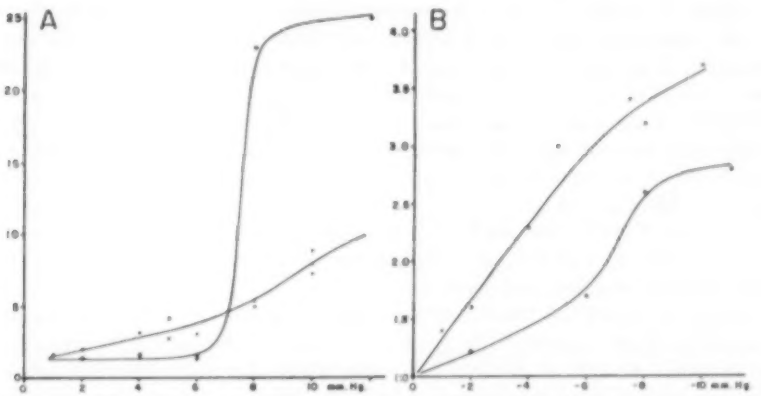

FIG. 2. Curves showing the relationship of stimulus intensities and magnitude of responses to positive pressures in A and negative pressures in B.

The dots correspond to the control conditions. The crosses are the values obtained when the oxygen tension in the inspired air was reduced to 10 per cent.

Abscissae: pressure applied in mm. of mercury (Hg.).; ordinates: relation between integrated respiratory activity during the first cycle following the stimulus and the activity for the same period of time during the control conditions. In B, the reciprocal of this relation was the one plotted.

After completion of one of these curves for the control conditions, a change was made and a whole curve was then obtained. It may be noted that these curves, in order to have an exact measure of the pressure applied, were obtained by closing the third tracheal opening. Thus, the stimulus was superimposed with some degree of asphyxia, the effects of which were controlled by taking a measurement with the pressure system at 0 mm. of mercury, from time to time, and subtracting these measurements from the values obtained with the pressures applied.

Effects of anesthesia: The surgical level of anesthesia was found to have very little effect either on the threshold or the magnitude of the responses to inflation or deflation of the lung. Of course, this is only an extrapolation, but the writer believes that, if the animal's state of anesthesia were allowed to become very light, the responses would not show any increase. On the other hand, with too much anesthetic (more than 60 mg. per kg.), the threshold rose and the magnitude of the responses decreased, except for positive pressures of relatively large intensities. In these last cases, the respiration was inhibited for longer times than in the control observations.

Effects of anoxia: In the animal breathing from a gas mixture in which the oxygen tension had been reduced below that in the normal air, the responses observed were very much the same as those obtained with too much anesthetic. A typical observation is illustrated in figure 2. It was necessary, however, to reduce the oxygen tension significantly (below 10 per cent) or to maintain a low oxygen saturation in arterial blood (less than 80 per cent) for many minutes in order to see an effect.

Breathing pure oxygen did not have any effect on the reflex responses.

Effects of carbon dioxide: A moderate increase in the carbon dioxide tension in the inspired air did not seem to have any effect on the relation of stimulus to response. It is true that inhibition of respiration with the inflation of the lung had a low absolute value and that the hyperpnea due to deflation was greater, but these changes were proportional to the increase in the basal respiratory activity. It is only necessary to multiply the absolute values obtained by the relation between the corresponding basal activities to have curves similar to those for the control period.

Decrease of carbon dioxide tension in the animal tissues, produced by previous hyperventilation, had the opposite effect on the absolute values. Again, the relation stimulus response was unaffected.

Effects of varying the number of stimulated receptors: The number of receptors stimulated could be reduced by tying a main bronchus or one of its branches, or by a partial or total crushing of the vagus nerves. In either case, the reflex responses thus obtained were correspondingly smaller. The responses obtained after occluding a main bronchus or after cutting one of the vagus nerves were reduced to about half those seen in the intact animal. Double vagotomy completely abolished the reflexes.

Opening of the chest cavity did not have any effect on the responses, even if, as was usual, the whole sternum and the anterior portion of the ribs were removed. The responses were dependent upon the state of inflation or deflation of the lung and not on the movements of the chest wall.

The Accomodation of the Reflexes

In another series of observations, the pressure was applied while the third tracheal cannula orifice remained open all the time. The animal was thus free to ventilate his lungs and there was no chance to create artificial conditions either of asphyxia or of hyperventilation. Under these conditions, the manometer reading cannot give an exact measure of the intrapulmonary pressure; the results, therefore, were not used for comparison with those described in the previous section, but were used just to follow the changes in the responses with time.

It was observed that, with the application of either positive or negative pressures, the frequency response soon reached an equilibrium. The curves illustrated in figure 3 show typical examples. In this particular case, the stimulus was maintained for only three minutes. Stimulation for longer periods of time did not change that picture (figure 4). Of particular interest is the fact that the ventilation of the lungs was equally changed in the same direction, and maintained at the new level for the entire period of stimulation, which in some cases was more than one hour. In figure 4 is illustrated the change in oxygen saturation of arterial blood after prolonged stimulation. A strong positive pressure could maintain the inhibition of the respiration in such a way that even the apparent signs of hypoxia were not enough to counteract the reflex inhibition (figure 5).

Another interesting point is that suppression of the stimulating pressure did not only bring to an end the corresponding reflex response. The change actually

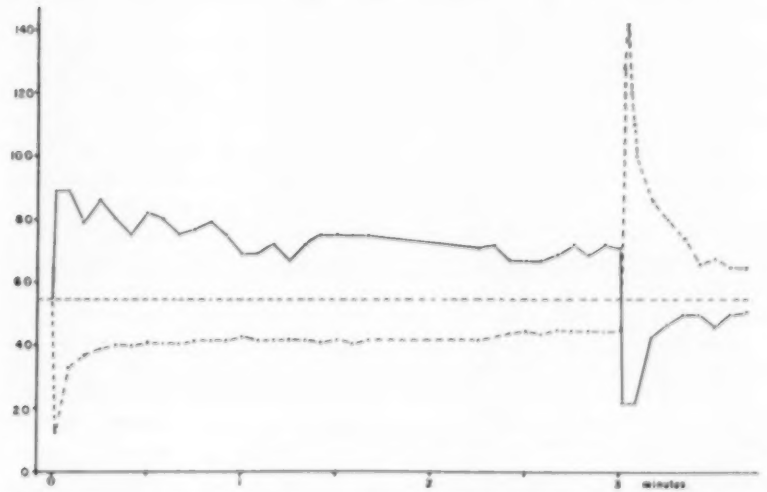

FIG. 3. Changes in the Hering-Breuer reflex responses with time. Stimulus applied with the tracheal cannula open.

Broken line: positive pressure; continuous line: negative pressure of the same value. Note that an equilibrium was reached very quickly, and that the "off" response at the end of a three-minute period of stimulation consisted of a change in the opposite direction.

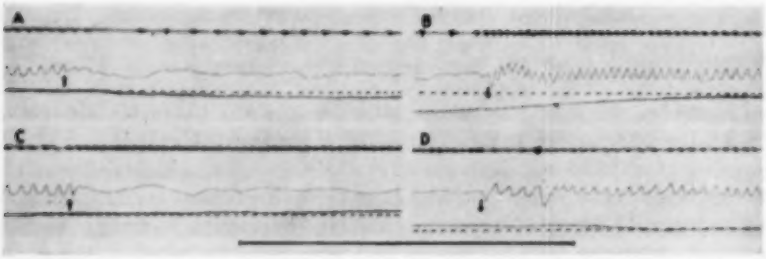

FIG. 4. Reflex regulation of pulmonary ventilation. Explanation of records is the same as for those in figure 1.

A. A positive pressure was applied at the arrow in an animal with the tracheal cannula open. The decreased respiratory activity was maintained for the entire period of stimulation (ten minutes in this case).

B. The end of the stimulus is indicated by the inverted arrow. There was a sudden change that quickly came to a new equilibrium, but at a different level from that of the control.

C and D. The corresponding records for a negative pressure stimulation.

The broken line is the reference line, the normal value in oxygen saturation of the arterial blood if the stimulus had not been applied.

Time calibration: thirty seconds.

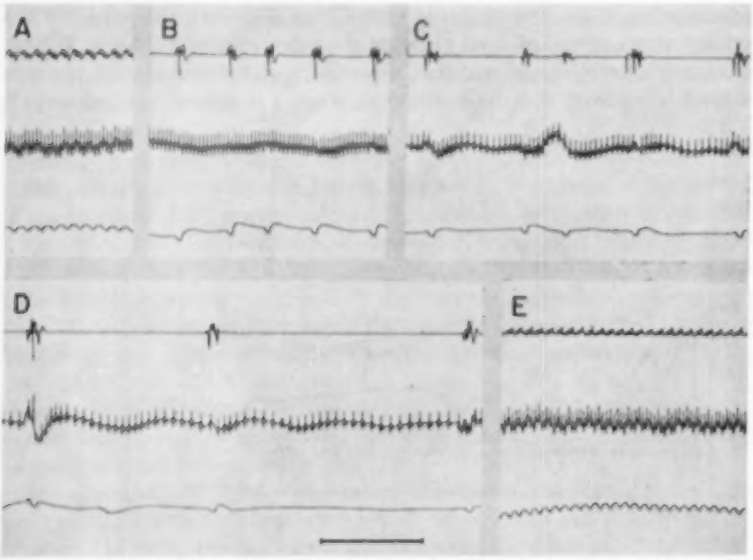

FIG. 5. Reflex response to inflation superimposed with asphyxia. Explanation of records is similar to that for figure 1. The middle record is an EKG.

A. Control.

B. After a positive pressure applied (10 mm. of mercury) with the trachea occluded.

C. Two minutes after application of positive pressure. Asphyxia of the animal is indicated by the slowing of the heart and the reduction in the amount of excitation in the respiratory center.

D. Three minutes later. Note the severe condition of asphyxia.

E. After removing the pressure. A new equilibrium had been established, this time in hyperventilation.

observed was precisely in the opposite direction. In other words, at the end of an applied positive pressure, an increased respiratory activity appeared well above the control level. The changes in blood oxygen saturation were not enough to explain this phenomenon. With applied pressures of increasing duration, the change became more and more prominent until, with stimuli of several minutes' duration, the "off" response was equivalent to the application, during the control period, of a stimulus of the same magnitude but opposite in size (*see* figure 3).

DISCUSSION

Most papers dealing with the Hering-Breuer reflex consider only the changes in breathing rate as a measure of the activity of this reflex. As Widdicombe (3) points out, this criterion is convenient for some purposes but, as it does not take into account tidal volume and respiratory muscle tone, it cannot be regarded as a good index of Hering-Breuer reflex activity.

In the present study, the actual pulmonary ventilation was not measured directly. For short periods of time, however, the records obtained with the thermocouple inserted in the tracheal cannula are, at least theoretically, proportional to that value. They give the rate of cooling during inspiration and the rate of warming during expiration, and this is proportional to the velocity of air flow. As the tracheal cannula is a tube with a constant cross section, at any given moment the rate of flow will be proportional to the amount of air flowing. Therefore, if one can eliminate the sources of error, such as changes in the temperature of the animal or the atmospheric air, or in the tracheal cannula itself, the records obtained will be a good index of the changes in lung ventilation. Unfortunately, the limitation of this method occurs precisely when the stimulus is applied. If the trachea is open, there will be a continuous flow of cool air from the stimulating system to the outside, or vice versa. If the trachea is closed, the actual movement of air through the cannula will be very small. In this last case, it is evident that ventilation will be almost zero.

When the tracheal cannula is open to the outside, a good criterion for changes in ventilation is the actual determination of oxygen saturation in arterial blood.

By recording the electrical activity of the diaphragmatic muscle, one can follow changes in muscle tension. There is evidence that electrical activity of the diaphragm is a very good indication of breathing activity, at least for the rabbit under anesthesia (4). This type of correlation between electrical activity and tension developed has been found to be true for other kinds of muscles (5).

All types of measurement used were found to complement each other and give the same kind of information.

The methods adopted here of determining the whole curve of respiratory activities under different pressures and of alternating a positive with a negative pressure reduced to a minimum the actual possible deviations in these kinds of measurement.

Adrian (6) has already shown that the responses of the pulmonary receptors are practically unaffected by decreasing the oxygen or increasing the carbon dioxide tensions in the inspired air. It can be concluded, therefore, that, if a

change in the reflex responses is observed by this mechanism, it must be due to an effect upon the integrating centers of respiratory activity. The facts that an increase in carbon dioxide tension does not have any appreciable effect and that a great decrease in oxygen tension is necessary to produce some change in the Hering-Breuer reflex responses (*see* figure 2) support the idea presented by many writers of the duality of the central mechanisms (7).

The main points it is important to emphasize are that the Hering-Breuer reflex can control respiratory activity (*see* figures 1, 3, and 4) even in conditions of severe asphyxia (*see* figure 5), and that the response soon comes to an equilibrium, which can last indefinitely (*see* figure 3), even if pulmonary ventilation is markedly shifted from the normal values (*see* figure 4). These points show that the organism can adapt itself to the changes of the gas tension in the blood more easily than to the reflexes under consideration.

Other data are available in the literature which show that organisms do adapt to changes in the tensions of the respiratory gases (8).

The present results can explain the clinical observation in which lung ventilation is seen to be impaired after partial pulmonary resection unaccompanied by thoracoplasty. The same may be said about the decortication of a lung that has been imprisoned by a thick pleura for a long time. In these cases, the remaining or the entire lung must be distended to occupy a greater volume. Another possible role of these reflexes is in lung emphysema or asthma. If the loss of elasticity and destruction of zones of lung tissue, or any other cause, produces some degree of distention of the rest of the alveoli, that distention can induce, reflexogenically, a decrease in respiratory activity of the muscles. The observation that emphysematous patients sometimes show an improvement when their lungs are partially collapsed, as during pneumoperitoneum, gives additional support to the idea proposed.

SUMMARY

In rabbits anesthetized with pentobarbital sodium (Nembutal®), the respiratory responses to inflation or deflation of the lung were measured by integrating in time the ventilation recorded by means of a thermocouple inserted in the tracheal cannula and the electrical activity of the diaphragmatic muscle. Changes in ventilation were also followed by recording the oxygen saturation in arterial blood.

The results show: that there is a definite relationship between stimulus and response which can be used for quantitative studies (*see* figure 2); that this relationship does not change with anesthesia up to the surgical level or with moderate increase or decrease of oxygen or carbon dioxide tension in the inspired air. Only with severe reductions in oxygen tension is there a rise in threshold and more marked inhibition to inflation of the lung with large positive pressures; the Hering-Breuer reflex can maintain a level in pulmonary ventilation for indefinite periods of time (*see* figure 3) even if it appears to be a condition of hyperventilation or of asphyxia (*see* figures 4 and 5); the organism adapts itself more easily to changes of the gas tensions in the blood than to the Hering-Breuer reflex.

The possible importance of this reflex in some clinical conditions is emphasized. Lung resection, when not followed by a corresponding thoracoplasty, leaves the remnant tissue to become distended to fill the space; this state would induce a reflex inhibition of the respiratory muscles, a fact which actually has often been reported. Asthma and emphysema are other diseases in which the Hering-Breuer reflex might play a part.

SUMARIO

El Reflejo de Hering-Breuer y su Importancia en el Control de la Respiracion

En conejos anestesiados con pentobarbital sodico Nembutal® se estudiaron las respuestas respiratorias a la distensión y al colapso del pulmón, integrando en el tiempo la ventilación registrada por medio de una termopila insertada en la cánula traqueal y la actividad eléctrica del diafragma. Tambien se siguieron los cambios en la ventilación, en los registros de la saturación de oxígeno de la sangre arterial.

Los resultados obtenidos muestran: Que hay cierta relación entre la intensidad del estímulo y la magnitud de la respuesta que puede ser usada en los estudios cuantitativos. (figura 2); Que esta relación no cambia con el grado de anestesia hasta el nivel quirúrgico, tampoco con cambios moderados de las tensiones del oxígeno y del bioxido de carbono en el aire inspirado. Con la reducción acentuada en la tensión de oxígeno hay aumento del umbral y mayor inhibición por la distensión del pulmón con presiones positivas elevadas; Que el reflejo de Hering-Breuer puede mantener un nivel de ventilación pulmonar por tiempo indefinido. (figura 3) aun cuando haya signos evidentes de hiperventilación o de asfixia (figuras 4 y 5); Que el organismo se adapta más facilmente a los cambios en la tensión de los gases de la sangre que al reflejo de Hering-Breuer.

Se señala la posible importancia de este reflejo en algunas condiciones clínicas. En las resecciones del pulmón, si no van seguidas de la toracoplastía correspondiente, el resto del pulmón es distendido para llenar la cavidad y esto puede inducir la inhibición refleja de los músculos respiratorios que a menudo ha sido descrita. Otros casos en que el reflejo de Hering-Breuer puede desempeñar cierto papel son el asma y el enfisema pulmonar.

RESUME

Les réponses réflexes à l'inflation ou à la déflation des poumons et leur importance dans la régulation de la respiration

Chez les lapins anesthésiés au pentobarbital sodique (Nembutal®), les réponses de la ventilation à l'inflation ou à la déflation du poumon ont été déterminées en intégrant dans le temps la ventilation, enregistrée à l'aide d'un couple thermoélectrique inséré dans la sonde trachéale, et l'activité électrique du diaphragme. Les variations de la ventilation ont été également suivies en enregistrant la saturation du sang artériel en oxygène.

Les résultats montrent: qu'il y a une corrélation définie entre le stimulus et la réponse à l'excitation qui peut être utilisée pour les études quantitatives (Fig. 2); que cette corrélation n'est pas modifiée par un degré d'anesthésie atteignant le niveau chirurgical ou par l'accroissement ou la réduction de la tension de l'oxygène ou du CO_2 dans l'air inspiré. L'élévation du seuil et une inhibition plus accentuée de l'inflation du poumon, sous l'influence de pressions positives élevées ne se manifestent que lorsque les réductions de tension de l'oxygène sont importantes. Le réflexe Hering-Breuer peut maintenir la ventilation pulmonaire à un certain niveau pendant des périodes de temps indéfinies (Fig. 3), même en présence d'hyperventilation ou d'asphyxie (Fig. 4-5). L'organisme s'adapte plus facilement aux variations de tension des gaz dans le sang qu'au réflexe Hering-Breuer.

L'accent est mis sur l'importance que peut avoir ce réflexe dans certains états cliniques. Les résections pulmonaires, non suivies de thoracoplastie correspondante, laissent en place un tissu résiduel qui se dilate et occupe l'espace libre. Cette condition étant apte à

provoquer une inhibition réflexe des muscles respiratoires, phénomène déjà souvent rapporté. L'asthme et l'emphysème sont d'autres cas dans lesquels le réflexe Hering-Breuer pourrait jouer un rôle.

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CORRELATION OF BIOLOGIC PROPERTIES OF STRAINS OF MYCOBACTERIUM WITH THEIR INFRARED SPECTRUMS[1, 2, 3]

IV. Fractionation and Comparison of the Lipids of Human Strains of *Mycobacterium Tuberculosis* by Means of Their Infrared Spectrums

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INTRODUCTION

It has previously been shown by means of infrared spectroscopic examination of extracts and fractions prepared from tubercle bacilli that chemical differences exist between various strains of mycobacteria known to be biologically distinct. Reproducible chemical differences were shown to exist between the H37Rv and H37Ra strains of *M. tuberculosis* (1), even when the extracted lipids were known to be complex. Tubercle bacilli of the avian variety could be differentiated from the mammalian varieties, again using a crude but reproducible extract (2). The chemical differences existing between human and bovine strains were not of the same order of magnitude; consequently, their recognition depended upon a more complete resolution of the extracted lipids. This was ultimately accomplished by means of column chromatography (3).

In continuing this work it has seemed of interest to the present writers to apply the techniques of column chromatography and infrared spectroscopy to other problems in the same general field. Observed variations in the resistance of strains of *Mycobacterium tuberculosis* to alcohol of different concentrations (4–6), streptomycin (7), and other agents (8) have suggested that tubercle bacilli of human origin might be subdivided, perhaps on the basis of chemical differences.

The purpose of the work described in the present paper is to find out to what extent a number of human strains of *M. tuberculosis*, known to be virulent for guinea pigs, may differ chemically. This was done by fractionating the bacterial lipids by column chromatography and by identifying the eluted compounds or mixtures by their infrared spectra.

MATERIALS AND METHODS

The cultures used for this investigation were all recent isolates from sputum with the exception of strains 225 (NIH 199RB) and 265 (H37Rv). Two of the recently isolated strains

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[1] From the Department of Medical Microbiology, University of Wisconsin; and the Harrison M. Randall Laboratory of Physics, University of Michigan.

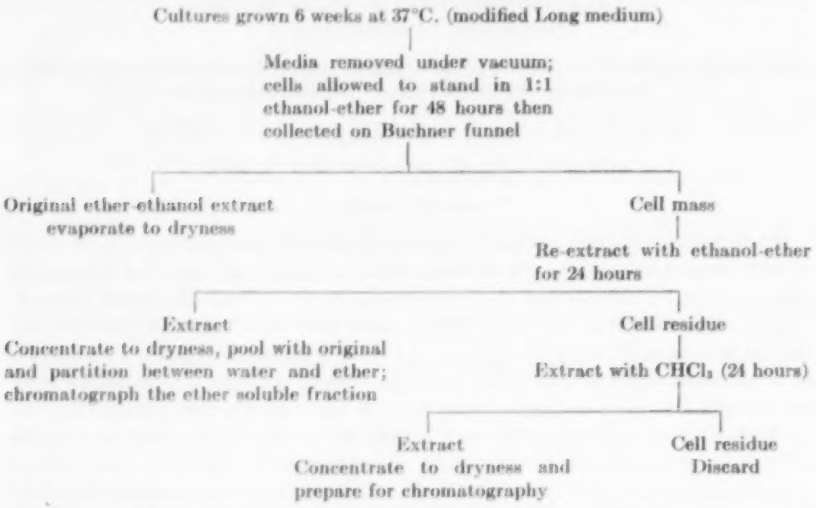
[2] Presented in part before the Medical Session, as part of *Section 3B*, at the annual meeting of the National Tuberculosis Association, Milwaukee, Wisconsin, May 24, 1955.

[3] This investigation was supported in part by research grants from the National Microbiological Institute (E-646), National Institutes of Health, Public Health Service; the Research Committee of the Graduate School from funds provided by the Wisconsin Alumni Research Foundation; the Rackham Fund; Graduate School, University of Michigan; and the Veterans Administration (Contract V1001 M-1636), Washington, D. C.

[4] Present address: Tuberculosis Laboratory, Communicable Disease Center, Chamblee, Georgia.

TABLE 1

FLOW DIAGRAM FOR THE EXTRACTION OF LIPIDS OF THE TUBERCLE BACILLUS


Cultures grown 6 weeks at 37°C. (modified Long medium)
Media removed under vacuum; cells allowed to stand in 1:1 ethanol-ether for 48 hours then collected on Buchner funnel
Original ether-ethanol extract evaporate to dryness
Cell mass
Re-extract with ethanol-ether for 24 hours
Extract
Concentrate to dryness, pool with original and partition between water and ether; chromatograph the ether soluble fraction
Cell residue
Extract with CHCl3 (24 hours)
Extract
Concentrate to dryness and prepare for chromatography
Cell residue
Discard

came from G. Canetti at the Pasteur Institute, Paris, France; two from A. T. Wallace, Edinburgh, Scotland; and the remaining eight, from several hospital laboratories in the United States. As mixed cultures of tubercle bacilli have been reported by various investigators (9–10), all strains were passed through a series of single-colony isolations. These isolations were accomplished by first growing the cells in liquid Tween®-albumin medium and then plating suitable dilutions on oleic-acid–albumin agar. The organisms were grown in mass culture on modified Long medium (11) and were incubated at 37°C. for six weeks. After incubation the medium was withdrawn under vacuum and discarded, and the cell mass was extracted directly according to the flow diagram summarized in table 1.

After testing several adsorbents, Magnesol®[5] was chosen and chromatographic columns (50 by 900 mm.) were packed with 85 to 100 gm. of a mixture of one part Magnesol® and one part Celite®.[6] The fraction to be chromatographed was dissolved in petroleum ether (60 to 75°C. boiling range) and was applied to the top of the column. The columns were sequentially developed with 1,800 ml. amounts of petroleum ether, petroleum ether-benzene (50-50), benzene, benzene-ether (50-50), and various mixtures of methanol in chloroform. The columns were treated as adsorption-elution chromatograms and the eluates (250 to 300 ml.) were collected as they dripped from the columns. The eluates were evaporated to dryness and the infrared spectra of the residues were recorded. Samples suspected of being complex were re-chromatographed on smaller diameter columns (13 by 900 mm.), packed with 15 to 18 gm. of adsorbent, and 10 ml. eluates were collected.

RESULTS

A few of the many infrared spectra obtained of free mycolic acids may be seen in Plate I. Figure 1 is the spectrum of pure alpha mycolic acid from Dr. Edgar

[5] Obtained from the Westvaco Chlor-Alkali Division, South Charleston, West Virginia.
[6] Celite Analytical Filter Aid was obtained from Johns Manville Company.

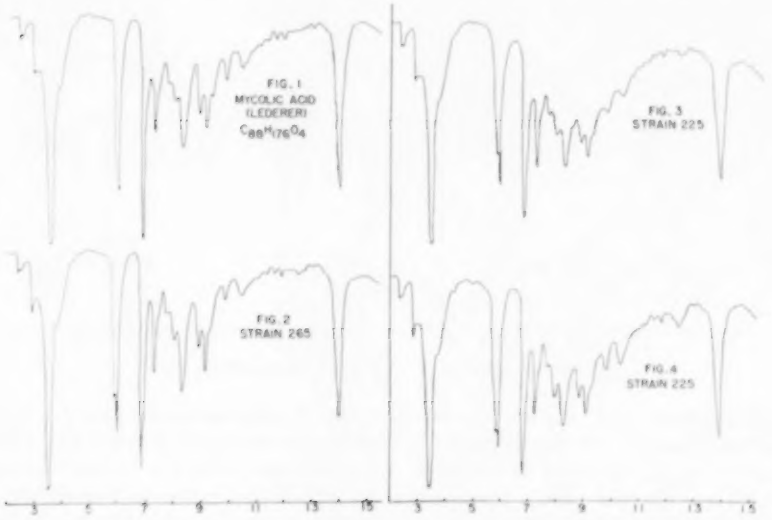


PLATE I. Spectral comparison of column-eluted mycolic acids with pure mycolic acid

Lederer (Paris). Figures 2–4 are the spectra of this acid eluted from three different chromatographic columns. Not only were the spectra of the three eluates obtained from three columns set up at different times, but the lipid mixtures applied to the columns were also somewhat different. Thus for figure 2 the mixture applied to the column (charge) was the total lipids extracted from the tubercle bacillus (strain 265) in a mixture of equal parts of methanol and chloroform. For figure 3 the charge was the ether-soluble portion of the ethanol-ether extract of strain 225, while for figure 4 the charge was from the total chloroform extract of the same strain. These charges of varying chemical content resulted in eluates of different composition except when they were pure compounds or, possibly, mixtures of constant content. The remarkable similarity of the spectra on Plate I indicates the excellent resolving power of the 50 mm. columns.

It is interesting to note that there is a variation in the amount of free mycolic acid found in the lipid extracts of various strains. It may be that this variation is correlated with the length of time a culture has been maintained on laboratory media, since strain 265 (H37Rv), actually maintained for fifty years on laboratory media, has been shown to contain 10 per cent free mycolic acid in the ethanol-ether soluble lipids in five distinct experiments. Likewise, strain 225, maintained for fifteen years on laboratory media, has been shown to contain from 2 to 4 per cent of free mycolic acid in the ethanol-ether soluble lipids while strain 244, isolated some five years ago, contains approximately 0.5 per cent of this acid. The fact that mycolic acid has been shown to exist as the free acid in some strains of tubercle bacilli is a confirmation of Lederer's work (12).

In Plate II are shown two spectra of a compound believed to be the salt of

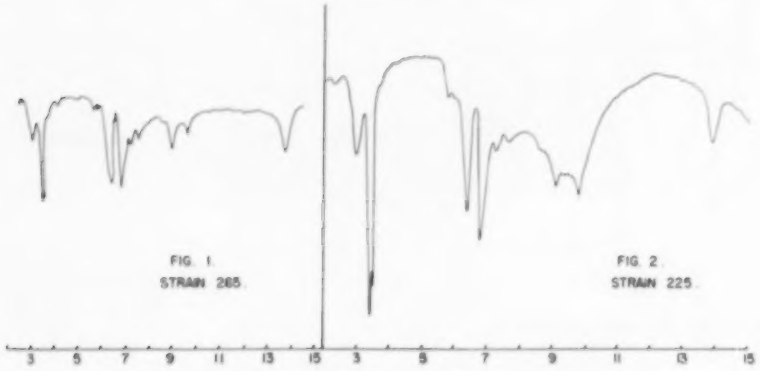


PLATE II. Spectra of salts of alpha mycolic acids

alpha mycolic acid. Figure 1 is the spectrum of this compound eluted from a column charged with the methanol-chloroform soluble lipids of strain 265, while figure 2 is the spectrum of the same compound eluted from a 13 mm. column which was charged with a fraction (strain 225) eluted earlier in benzene-ether from a 50 mm. column.

These spectra show in the intense band at 6.4 mu the characteristics of an ionized carboxylate group, COO^-. The sample, when treated with ether saturated with hydrogen chloride gas, gives the spectrum of alpha mycolic acid, indicating the conversion of the salt to its free acid. The spectra of Plate II for this reason are labeled salts of alpha mycolic acid. Thus far, this compound has been found in a pure state only in strains 225 and 265. In a limited number of chromatographic separations of the chloroform soluble waxes, however, this salt has appeared in impure form in strain $244S_2$.

It should be noted that, when the total lipids soluble in methanol-chloroform are cooled to 5°C. in this solvent pair, a white amorphous solid separates. This 5°C. insoluble fraction from strain 265 shows the band at 6.4 mu, along with other strong bands, and this band can also be made to disappear by treatment with HCl in ether. Accordingly, it would seem that the salt is normally present in this strain and that the chromatographic column is not responsible for its presence.

Figure 1 of Plate III is the spectrum of pure dimycoceronate of phthiocerol as acquired from Dr. Lederer, while figures 2–4 are examples of some of the spectra of the compound eluted from columns. The similarity of these four spectra is very marked, leaving no doubt of the identity of the eluted compounds. One variation that has resulted from the passage of cultures through a series of single-colony isolations is seen in strain $244S_2$. This strain differs from the parent strain 244-O ("O" for original) in that the ether soluble portion of the ethanol-ether lipids does not contain measurable quantities of the very prominent dimycoceronate of phthiocerol. This compound makes up 10 to 15 per cent of the lipids of all other strains studied thus far.

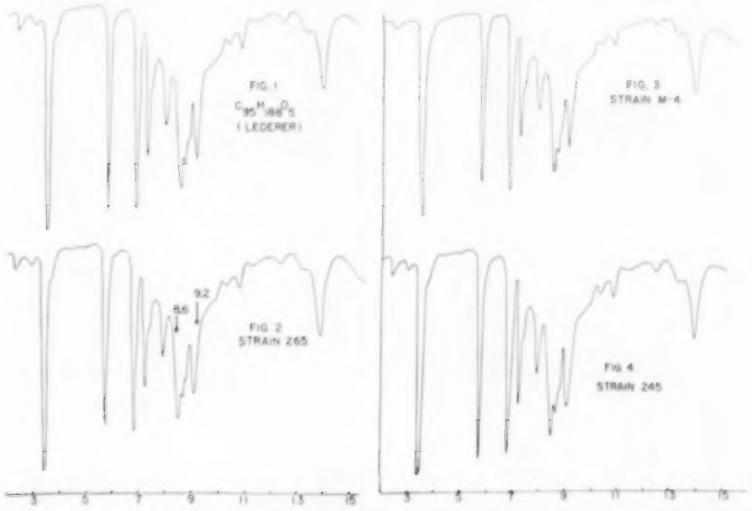


PLATE III. Comparison of spectra of dimycocerenate of phthiocerol type of compounds

The infrared spectrum of crystalline films of aliphatic or straight-chain saturated fatty acids is very characteristic and easily recognized. An identifying region of the spectrum is the region between 7.7 and 8.6 mu (Plate IV); in this region a series of absorption bands appear which have been designated as "fine" or "feather" structure. Sinclair and associates (13) have made extensive studies of this phenomenon and state that the fine structure is due to methylene ($—CH_2—$) wagging and/or twisting vibrations. The fine structure is absent from spectra recorded for solutions or melts (samples heated above their melting point when the spectrum is recorded) of fatty acids. The number and separation of the fine structure bands are the function of the length of the hydrocarbon chain (14, 15). To date it has not been possible to get a collection of the various types of long-chain fatty acids likely to be found in the lipids of this organism. It has not been possible to make a comparison of the spectra of the eluates containing this characteristic fine structure with spectra of pure compounds of known composition. The fine structure separation of the fatty acids and esters in these lipid extracts has varied from 0.085 to 0.135 mu, but sufficient data are not yet available to justify attempts at interpretation of these separations. For comparison of strains, therefore, only the presence or absence of this fine structure is used, with no attempt at identifying the fatty acids concerned. Examples of spectra containing such fine structure are recorded in all figures of Plates IV and V and in figures 1–3 of Plate VI.

The isolation of mycolic acid and the dimycoceronate of phthiocerol in a form spectroscopically identical to the same compounds isolated by Lederer suggests that other compounds in a more or less pure state may also be found among the

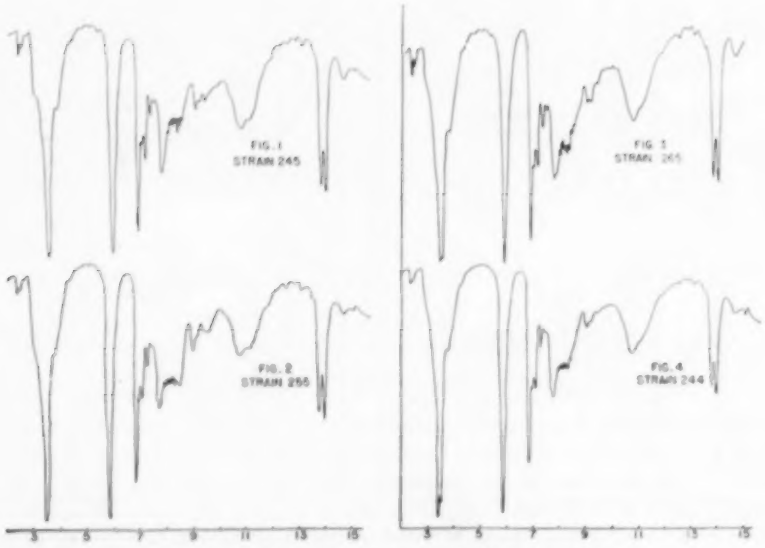


PLATE IV. Spectra of column-eluted fatty acids

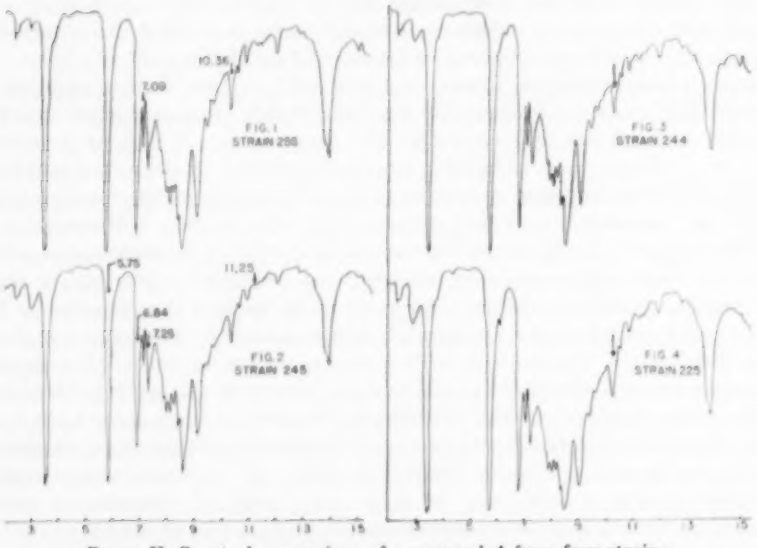


PLATE V. Spectral comparison of compound *A* from four strains

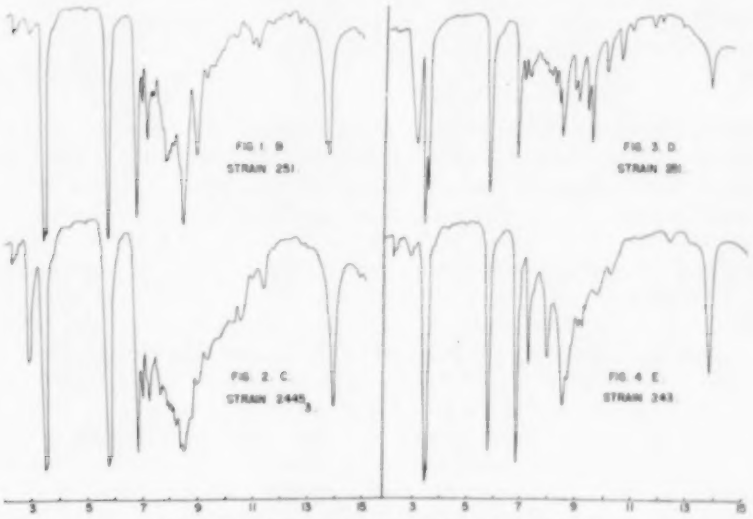


PLATE VI. Spectra of compounds *B*, *C*, *D*, and *E*

samples eluted from the columns. Other substances are thought to be relatively pure compounds or, possibly, mixtures of constant composition when a given spectrum is accurately repeated in eluates from several strains under different conditions of experimentation. The spectra of one such unknown component are shown in Plate V. This component has been repeatedly isolated from every strain thus far examined and its spectrum is so characteristic and reproducible that it is believed that this component, called compound *A*, is either a relatively pure compound or a mixture of constant composition. It may be suggested, from its infrared spectrum, that compound *A* is a branched-chain, unsaturated fatty acid ester having the *trans* configuration at the double bond.

Plate VI contains single examples of the spectra of eluates believed to be relatively pure compounds or mixtures of constant composition. In either case, the spectra may be used in characterizing the strains in which they occur. The belief that these eluates are relatively pure compounds or fixed mixtures rests mainly upon their regular occurrence in repeated tests which were made with each strain. This evidence is strengthened, first, by the fact that the column charges in these tests on individual strains have varied in the manner indicated earlier in discussing the known compounds; and, second, by the fact that in several instances the compounds appeared when the columns were packed with different adsorbents such as alumina, silica gel, or silicic acid.

Using the infrared spectra of the known and provisionally recognized compounds as a means of identification, a very fair characterization of the fourteen strains which have been studied at all intensively can be achieved. It should be remarked that both of the techniques used, chromatography and infrared spectroscopy, are capable of development for quantitative work. By the quantitative

methods, the strains could be characterized by the amounts of any compound found as well as by its presence or absence.

DISCUSSION

From the results presented in this paper, it appears that it is possible to characterize the various strains of *M. tuberculosis* by the chemical content of their lipid extracts. Whether the chemically distinct strains also differ biologically remains to be determined. Nevertheless, a method capable of detecting chemical variations between strains which are presumably as similar as these should prove valuable in an examination of "atypical acid-fast bacilli" and their relationship to tuberculosis in man.

With this background of information on the chemical content of the lipids of fourteen human strains of tubercle bacilli and the data that are being accumulated on avian bacilli, strains of bovine origin, and some of the saprophytes (*M. phlei*, *M. smegmatis*, and *M. butyricum*), it should be possible by means of an analysis of this type to make contributions to an understanding of the problem.

For example, it may be possible to decide whether these atypical strains are more closely related to true human bacilli, perhaps being in some cases a drug-induced variant that is avirulent for guinea pigs. Conversely, it may be that this type of atypical organism more closely resembles the saprophytes or perhaps is chemically dissimilar to either the saprophytic or the pathogenic mycobacteria. Then, assuming the origin of the strain to be known, it would be valuable to have a rapid simple method of determining whether a given strain is "atypical," human-virulent, or saprophytic.

SUMMARY

Through the use of chromatography and infrared spectroscopy, the lipids of fourteen strains of tubercle bacilli of human origin have been examined. By noting the presence or absence of a series of nine recognized compounds in each of the strains, it is possible to subdivide these strains on the basis of their chemical differences.

Presumably more extended experience will result in the isolation of more pure compounds together with better purification of those already isolated. These findings should permit both qualitative and quantitative distinctions to be made with greater accuracy.

SUMARIO

Correlación de las Propiedades Biológicas de las Cepas de Micobacterias con Sus Espectros Infrarrojos: IV. Fraccionación y Comparación de los Lípidos de Cepas Humanas del Mycobacterium Tuberculosis por Medio de Sus Espectros Infrarrojos

Por medio de la cromatografía y la espectroscopia infrarroja, se examinaron los lípidos de 14 cepas del bacilo tuberculoso humano. Notando la presencia o falta de una serie de nueve compuestos reconocidos en cada una de las cepas, es posible subdividir éstas a base de sus diferencias químicas.

Presuntamente una observación más extensa dará por resultado el aislamiento de más compuestos puros junto con la mejor purificación de los ya aislados. Estos hallazgos deben

permitir el establecimiento de distinciones tanto cualitativas como cuantitativas con mayor exactitud.

RESUME

Corrélation entre les propriétés biologiques des souches de Mycobactéries et leurs spectres infra-rouges: IV. Fractionnement et comparaison des lipides de souches humaines de Mycobacterium tuberculosis au moyen de leurs spectres infra-rouges

La chromatographie et la spectroscopie infra-rouge ont été utilisées pour l'examen des lipides de souches de bacilles tuberculeux humains. Dans chaque souche la présence ou l'absence d'une série de neuf composés reconnus a été notée; ceci a permis de subdiviser ces souches sur la base de différences chimiques.

Il est probable qu' une étude plus étendue permettra d'isoler un plus grand nombre de composés à l'état pur et améliorer la purification des déjà isolés composés. Ces résultats devraient permettre d'établir une distinction qualitative et quantitative plus précise.

Acknowledgments

Although Professor W. J. Nungester no longer participates in these investigations, he has maintained an active interest in them, which the writers appreciate. They wish also to thank him for the use of the double-beam infrared spectrograph from his department as well as for other of its facilities. They wish again to acknowledge their indebtedness to Mr. Harold Patterson for his skill in preparing the samples and for operating the spectrograph.

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CYCLOSERINE: ANTITUBERCULOUS ACTIVITY IN VITRO AND IN THE EXPERIMENTAL ANIMAL[1, 2]

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INTRODUCTION

The initial observations on the effectiveness of the new antimicrobial, cycloserine, were reported in February, 1955 (1), and a number of reports have subsequently appeared in the literature (2–9). The present report is concerned with studies of the *in vitro* bacteriostatic and bactericidal power of the drug against several mycobacterial strains, the emergence of cycloserine-resistant strains of tubercle bacilli, and the effect of the drug on experimental tuberculosis in guinea pigs and mice.

MATERIALS AND METHODS

Tuberculostatic and tuberculocidal activity in liquid media: The tuberculostatic and tuberculocidal properties of cycloserine[3] were investigated in liquid media, using as test organisms *M. tuberculosis* var. *hominis* H37Rv, its streptomycin- and isoniazid-resistant descendants, the bovine and avian strains 4228-4, and Sheard (fully virulent), and the saprophytic strain M. 607. The drug was dissolved in water in concentration of 10 mg. per ml., sterilized by filtration through sintered glass, and added to the test media to make twofold dilutions from 200 to 1.5 γ per ml. An inoculum of 0.1 ml. of a ten-day culture growing in Tween®-albumin liquid medium was introduced into each tube of 5 ml. of test medium and the tubes were incubated at 37° C. for twenty-eight days. A rough estimate of tuberculocidal effect was made by subculturing 0.15 ml. onto solid egg medium slants on the fifteenth day of incubation and by subcutaneous inoculation of guinea pigs with 1 ml. of material from the H37Rv tubes on the twenty-eighth day of incubation. The results are presented in table 1.

OBSERVATIONS

Studies in Vitro

It may be seen in table 1 that cycloserine concentrations of approximately 6.25 γ per ml. completely inhibited the growth of the strains of tubercle bacilli of human origin for fourteen days, whereas the one bovine strain tested required 12.5 γ and the avian strain 25 γ of drug per ml. for inhibition. The saprophytic *M.* 607 was much more resistant to the drug. The cycloserine concentration necessary to inhibit the H37Rv strains resistant to either streptomycin or isoniazid was the same as that for the parent susceptible strain. The presence of 10 per cent horse serum in Proskauer and Beck medium was accompanied by a

[1] From the Trudeau Laboratory, Trudeau Foundation, Inc., Turudeau, New York.

[2] This study was aided by a grant from the Division of Research Grants and Fellowships of the National Institutes of Health, Public Health Service.

[3] Cycloserine was generously supplied by Commercial Solvents Corp. (Lot 24) and Eli Lilly & Co. (Lot 43).

TABLE 1

BACTERIOSTATIC AND BACTERICIDAL ACTIVITY OF CYCLOSERINE IN LIQUID MEDIA

Test Strain	Medium	Minimal Inhibitory Concentration (γ/ml) at Given Incubation Time in Days				Minimal Bactericidal Concentration*
		7	14	21	28	(γ/ml)
H37 Rv	Tween®-albumin	6.25	6.25	6.25–12.50	12.5–25.0	200
	Proskauer and Beck	3.10	6.25	6.25	12.5	
	Proskauer and Beck with 10% horse serum	12.50	25.00	25.00	25.0–50.0	
H37 Rv resistant to streptomycin	Tween®-albumin	6.25	6.25	6.25	12.5–25.0	200
H37 Rv resistant to isoniazid	Tween®-albumin	6.25	6.25	12.50	12.5–25.0	200
Bovine 4228-4	Tween®-albumin	6.25	12.50	12.50	25.0	>200
Avian Sheard	Tween®-albumin	12.50	25.00	25.00	50.0	>200
M. 607	Tween®-albumin	100.00†	—	—	—	>200‡

* As determined by the absence of growth when, on the fifteenth day of incubation, 0.15 ml. from the original cycloserine tubes was inoculated on slants of solid medium.

† 50 γ per ml. from two to five days.

‡ On ninth day of incubation.

fourfold decrease in the activity of the cycloserine. After twenty-eight days of incubation, growth of tubercle bacilli appeared in fourfold higher concentrations of cycloserine than had been inhibitory during the first week.

On the fifteenth day of incubation, all cultures which contained less than 200 γ of cycloserine per ml. yielded growth on subculture. With the bovine, avian, and *M.* 607 strains, viable organisms were still present even at this concentration. Nevertheless, the number of viable bacilli was sharply decreased in the higher drug concentrations. This was evidenced by the fact that material from the cultures containing cycloserine concentrations of 12.5 γ per ml. gave a heavy confluent growth on subculture, whereas tubes with drug concentrations between 25 and 200 γ per ml. yielded only a few discrete colonies. The results of guinea pig inoculations were in agreement with these data obtained *in vitro*. Material from H37Rv tubes containing 12.5 γ or less produced extensive progressive tuberculosis, whereas animals which received material from tubes containing 25 to 100 γ of cycloserine per ml. showed only a small amount of slowly progressive disease three months after infection. Unfortunately, animal inoculation was not done from the tube which contained 200 γ of cycloserine per ml.

Growth inhibition in solid media: Several human, bovine, and avian strains were tested in drug-containing slants of inspissated egg medium (American Trudeau Society (ATS) medium) and Middlebrook's 7H-9 modification of oleic-acid–albumin agar (10). The minimal inhibitory concentration of cycloserine in ATS medium for 7 human strains (including 5 stock strains and 2 recently isolated from patients) was 20 γ per ml., but the bovine Ravenel and

TABLE 2

STABILITY OF AQUEOUS 1 MG. PER ML. SOLUTION OF CYCLOSERINE AT ROOM TEMPERATURE AS MEASURED BY TUBERCULOSTATIC ACTIVITY AGAINST H37Rv IN TWEEN®-ALBUMIN MEDIUM

Age of Solution	Minimal Inhibitory Concentration in γ/Ml. at Days of Incubation	
	14	28
2 hours	6.25	12.5
7 days	6.25	12.5
14 days	6.25	12.5
21 days	6.25	12.5
28 days	6.25	25.0
37 days	12.50	50.0

TABLE 3

APPEARANCE OF CYCLOSERINE-RESISTANT TUBERCLE BACILLI ON SERIAL TRANSFER IN TWEEN®-ALBUMIN MEDIUM, H37Rv STRAIN

Transfer Number	Highest Concentration Allowing Growth in 14 days
	γ/ml.
1	3.10
2	1.50
3–5	3.10
6	6.25
7–10	12.50
11	25.00
12	12.50
13	25.00

the avian Sheard strains required 40 γ per ml. In every instance, the minimal inhibitory concentration of cycloserine was twice as high in the agar medium as in the ATS medium. A few trials in Herrold's egg yolk agar gave the same results as those in the ATS medium.

Influence of pH on cycloserine activity: Proskauer and Beck liquid synthetic medium was adjusted to pH levels of 5.8, 6.4, 7.0, and 7.8 with either N sulfuric acid or 10N sodium hydroxide, and cycloserine was added in twofold dilutions. It was found that at pH 6.4 and 7.0, cycloserine concentrations of 5 γ per ml. were sufficient to inhibit growth of the H37Rv strain for fourteen days, while 10 γ were necessary at pH 7.8 and 20 γ at pH 5.8.

Drug stability: An aqueous solution of cycloserine containing 1,000 γ per ml. was kept at room temperature for thirty-seven days, and at intervals the antituberculous activity was determined by making serial twofold dilutions into tubes of Tween®-albumin medium and inoculating with the H37Rv strain. From the results in table 2 it may be seen that there was no significant change in antituberculous activity for twenty-one days, after which a detectable deterioration occurred.

Appearance of drug-resistant strains: The H37Rv strain of *M. tuberculosis*

TABLE 4

RESULTS OF CYCLOSERINE THERAPY IN GUINEA PIGS STARTING ONE WEEK AFTER INFECTION WITH H37Rv BY INTRACARDIAC ROUTE

Drug Administration Intramuscularly Once Daily

Treatment*	Survival in Days After Infection
0 (Controls)	30, 32, 38, 44, 55(K)†, 55(K)
Cycloserine 10–15 mg.	30, 31, 39, 45, 55(K)
Cycloserine 20–25 mg.	23, 29, 34, 36, 55(K)
Cycloserine 40–50 mg.	29, 34, 41, 46, 55

* After 28 days of treatment (35 days after infection), dosage was increased to 100 mg. daily for all surviving animals.

† (K) indicates animal sacrificed.

was transferred serially at two-week intervals in Tween®-albumin medium containing increased concentrations of cycloserine in an attempt to derive a strain highly resistant to the drug. The results are shown in table 3. There was no decrease in susceptibility during the first five transfers, but twofold increases in resistance were noted after the sixth, seventh, and eleventh transfers. This pattern is similar to that noted with *p*-aminosalicylic acid (PAS), and stands in contrast to the more rapid and abrupt changes seen with isoniazid and streptomycin.

Studies in Vivo

Mice: Eighteen C57 black mice were infected intravenously with 0.3 mg. (dry weight) of H37Rv tubercle bacilli. Six mice remained untreated and 12 were treated with cycloserine, starting five days after infection and continuing for twenty-six days. The drug was administered subcutaneously once daily in freshly made aqueous solution. Six mice were given 1 mg. (40 mg. per kg. per day) and 6 mice were given 5 mg. (200 mg. per kg. per day). All survivors were killed on the thirty-second day after infection.

In agreement with the findings of other workers (2, 6, 11), no beneficial effect from cycloserine was observed on either the survival time or the extent of gross pulmonary disease in the experimentally infected mice.

Guinea pigs: Twenty-one guinea pigs, weighing approximately 700 gm. each, were infected with 0.1 mg. (dry weight) of H37Rv tubercle bacilli by the intracardiac route. Six remained untreated and 15 were given a freshly made aqueous solution of cycloserine intramuscularly once daily, starting one week after infection. Three groups of 5 animals each received 10, 20, or 40 mg. of cycloserine per day, respectively. The dosage was increased after eighteen days to 15, 25, and 50 mg., and after a further ten days to 100 mg. of cycloserine for all surviving animals. By this time the animals had lost weight to below 500 gm., and the latter dose represented at least 200 mg. per kg. per day. The survivors were sacrificed on the fifty-fifth day of infection.

The results of these experiments in guinea pigs are indicated in table 4, from which it may be seen that there was no significant beneficial effect from the cycloserine treatment in any dosage. All animals which succumbed showed progressive tuberculosis, although many which died early in each of the treated

groups revealed enlarged pale livers with irregular gray-yellow areas. Histologic study of the organs has not as yet been completed. These negative results parallel those of Patnode and associates (8) obtained from guinea pigs infected with tubercle bacilli by the subcutaneous route.

Blood cycloserine concentrations: Biologic assays of the cycloserine concentrations in the blood of treated guinea pigs were made by a cylinder plate method using the *Staphylococcus aureus*, strain 209P. After a single intramuscular dose of 50 mg. of cycloserine (65 to 70 mg. per kg.), the average plasma concentrations in 2 normal guinea pigs were 95 γ per ml. at one and one-half hours, 50 γ at three and one-half hours, and 10 γ per ml. at seven hours. One normal guinea pig given 100 mg. of drug intramuscularly (170 mg. per kg.) showed a plasma concentration of 400 γ per ml. at one hour, and 100 γ at three and one-half hours after administration of the drug.

DISCUSSION

The reasons for the relatively low effectiveness of cycloserine in the therapy of experimental tuberculosis of guinea pigs and mice are as yet unknown. Blood concentrations of biologically active material in the guinea pig were many times greater than the minimal concentration necessary for inhibition *in vitro*, and, since the drug diffuses readily throughout the body fluids (2, 3), it may be assumed that comparably high concentrations of drug were likewise present in the tissues. It will be remembered, however, that in the presence of 10 per cent horse serum the bacteriostatic concentration of cycloserine was increased four-fold to approximately 25 γ per ml., and Cummings (4) reported that 10 and 20 γ of drug per ml. were the inhibitory concentrations in the presence of 5 per cent human and guinea pig serum, respectively.

In contrast to the poor results in mice and guinea pigs, it has been found that the administration of cycloserine in dosage of at least 200 mg. per kg. per day produced definite, but limited, beneficial effect in experimental tuberculosis of rabbits (14) and monkeys (15). The factors responsible for this difference in response demand investigation, and the situation clearly demonstrates the importance of using several animal species for the evaluation of potential antituberculous agents.

Strains of tubercle bacilli resistant to cycloserine appear much more slowly after repeated transfers in liquid medium than is the case with streptomycin or isoniazid. This should not be taken as an indication, however, that cycloserine-resistant strains of tubercle bacilli will not be encountered in patients. It will be remembered that PAS-resistant strains of tubercle bacilli are difficult to derive in this manner and yet are frequently isolated from patients treated with PAS alone.

The stability of cycloserine has been studied more intensively in other laboratories. Cummings and associates (4), using a chemical assay procedure, reported a 34 to 55 per cent loss of the antituberculous activity of cycloserine in fifteen days in Proskauer and Beck medium at 37°C. The percentage loss was inversely proportional to the original drug concentrations in the range of 10 to 40 γ per

ml. Because of similarity in minimal inhibitory concentration in liquid medium and in modified Löwenstein solid egg medium, Cummings and associates concluded also that the drug is stable to the inspissation procedure, a finding with which the presently reported data are in agreement. The loss of "appreciable antituberculous activity" of cycloserine in oleic-acid–albumin agar after five days at room or refrigerator temperature has been noted by the Fitzsimons group (12). This may explain the finding of a higher minimal inhibitory concentration in agar medium than in the ATS medium as reported herein.

At the Livermore Veterans Administration Hospital it was found that a 20 γ per ml. aqueous solution of cycloserine (pH 7.03) was stable at 0 to 4°C., decomposed 8 per cent during an inspissation procedure, and showed progressive decomposition during incubation at 37°C. after inspissation (13). The Merck group reported the finding of instability of acidic and neutral solutions of cycloserine as contrasted with relative stability of basic solutions (6). It is probable that the fourfold "drift" of the minimal inhibitory concentration in liquid medium on prolonged incubation noted in the present report was due, at least in part, to deterioration of the drug.

SUMMARY

Cycloserine was found to inhibit the growth of human virulent tubercle bacilli *in vitro* in concentrations of 6.25 γ per ml. in liquid medium and 20 γ in solid (American Trudeau Society) medium. Strains of *M. tuberculosis* resistant to streptomycin or isoniazid showed no difference in susceptibility to cycloserine. There was a fourfold decrease in drug-susceptibility in the presence of horse serum. One bovine and one avian strain were slightly less susceptible to cycloserine than was the human strain which was studied. In the manner tested, a drug concentration of at least 200 γ per ml. was necessary to "sterilize," although a sharp decrease in the number of viable tubercle bacilli was noted at the presence of cycloserine concentrations of 25 γ per ml. The drug showed its greatest activity at pH 6.4 to 7. Mycobacterial resistance increased slowly in a strain transferred serially in liquid drug-containing medium. No beneficial effect from cycloserine was noted in experimental tuberculosis of mice infected intravenously and treated with 40 or 200 mg. per kg. per day, or in guinea pigs infected by the intracardiac route and given up to 200 mg. per kg. per day parenterally. Comparable doses of cycloserine given to normal guinea pigs produced maximal drug concentrations in the plasma of 95 to 400 γ per ml.

SUMARIO

Cicloserina: Actividad Antituberculosa in Vitro y en el Animal de Experimentación

La cicloserina se mostró capaz de inhibir la proliferación de bacilos tuberculosos humanos virulentos *in vitro* a una concentración de 6.25γ por ml. en medio líquido y de 20γ en el medio sólido de la Sociedad Americana Trudeau (ATS). Cepas resistentes a la estreptomicina o la isoniacida no revelaron diferencia en su susceptibilidad a la cicloserina. Cuando había suero de equino presente, se cuadriplicó la disminución de la sensibilidad. Una cepa bovina y una aviaria fueron un poco menos susceptibles que la humana a la droga. En la forma en que se hizo el ensayo, se necesitó una concentración a lo menos de 200γ por ml.

para "esterilizar," aunque con 25γ por ml. ya se notó una disminución notable en la cantidad de bacilos viables. La droga reveló su actividad máxima a un pH de 6.4 a 7. La resistencia micobacteriana aumentó lentamente en una cepa pasada en serie por un medio líquido que contenía la droga. No se notó ningún efecto beneficioso de la cicloserina en la tuberculosis experimental de los ratones infectados intravenosamente y tratados con 40 ó 200 mgs. por kg. al día o en los cobayos infectados por vía intracardíaca que recibieron hasta 200 mgs. por kg. al día parentéricamente. Dosis comparables de cicloserina administradas a cobayos normales produjeron concentraciones máximas de 95 a 400γ por ml. en el plasma.

RESUME

Cyclosérine: Activité antituberculeuse in vitro et dans l'expérimentation chez l'animal

La cyclosérine a démontré *in vitro* son activité inhibitrice sur la croissance du bacille tuberculeux virulent humain lorsque les concentrations atteignaient 6,25γ par ml. en milieu liquide et 20γ par ml. en milieu solide, milieu American Trudeau Society (ATS). Les souches streptomycino-ou isoniazide-résistantes n'ont présenté aucune différence de sensibilité à la cyclosérine. En présence de sérum de cheval la sensibilité est quatre fois plus réduite. Une souche bovine et une souche aviaire se sont montrées légèrement moins sensibles à l'antibiotique que la souche humaine. Dans les conditions de l'expérience, la "stérilisation" n'a été obtenue qu'avec une concentration d'au moins 200γ par ml., mais une diminution brusque du nombre de la population viable était observée à 25γ par ml. L'activité de la drogue atteignait son activité maxima de pH 6,4 à pH 7. La résistance mycobactérienne d'une souche augmentait lentement dans une souche par les transferts en série dans un milieu liquide renfermant la cyclosérine. La cyclosérine n'a pas été efficace dans la tuberculose expérimentale de la souris inoculée par voie intraveineuse et traitée avec une dose de 40 ou 200 mg./kg. par jour, ni chez le cobaye inoculé par voie intracardiaque et recevant 200 mg./kg. par jour par voie parentérale. Des doses comparables de cyclosérine administrées au cobaye sain produisaient des concentrations plasmatiques maxima de 95 à 400 γ par ml.

Addendum

Additional data on the stability of cycloserine in solid egg media have indicated that detectable loss of antituberculous activity had occurred after two weeks at room temperature, but not until after the fourth week when the drug-containing medium was kept at 4°C.

In the attempted derivation of a drug-resistant strain, the emergence of bacilli capable of growing in 60 γ of cycloserine per ml. of medium was observed during the fourteenth to twentieth transfers.

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THE SIGNIFICANCE OF ANTIBODIES TO TUBERCULOPROTEIN AND POLYSACCHARIDE IN RESISTANCE TO TUBERCULOSIS[1,2]

Interference with Antibodies by These Antigens

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INTRODUCTION

Antibodies to crude antigens of the tubercle bacillus, as well as some of its partially or highly purified components, have been studied repeatedly. The former have been used by many investigators in the complement-fixation test on human tuberculous serum, and there has been a certain degree of correlation between positive reactions and known tuberculous disease (1). In recent years numerous studies have been made on the correlation between infection and the hemagglutination reaction, as described by Middlebrook and Dubos (2). The specific antigen in this case has been thought by them to be a polysaccharide which was present in the crude Old Tuberculin antigen. Other data, however, indicate that protein also is involved.

When purified soluble antigens are available, the simple direct precipitin reactions can be used to detect antibodies and to determine whether they are antibodies to protein or to polysaccharide. Seibert was able to demonstrate consistently the presence of large quantities of protein antibodies in the serum of sensitized guinea pigs and rabbits, but found such antibodies only irregularly in human tuberculous serum (3). By means of the precipitin test the proteins made from tuberculins of human, bovine, and timothy bacilli could be differentiated (3), but the proteins from five different human strains of tubercle bacilli could not be distinguished from each other (4). Henderson (5) extended this method of differentiation to a whole series of acid-fast bacilli isolated from leprosy lesions. Menzel and Heidelberger (6), McCarter, Kanne, and Hastings (7), and Schaefer (8) also were able to distinguish between different acid-fast bacilli in this way. Later (9), with more highly purified protein antigens, it was possible to make some serologic distinction even between the different proteins isolated from a single strain of the human tubercle bacillus.

Antibodies to polysaccharide also have been recognized by many workers (10–14) in serum from animals sensitized by dead or living tubercle bacilli. With one very highly purified tuberculin polysaccharide, it was possible to stimulate antibodies (13) while, in most of the other studies, the polysaccharide antigen served merely as a haptene in the precipitin reaction. Some investigators

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using purified polysaccharide preparations have also found positive precipitin reactions with human serum. Kropp and Foley (15), Choucroun (16), and Seibert, Stacey, and Kent (13) have reported that antibodies to polysaccharide are present in some, but not in all, tuberculous serums tested. Choucroun tested the serum of 298 tuberculous patients and 99 normal or nontuberculous persons. Forty-one per cent of the tuberculous patients showed positive reactions, and she thought that the antibodies to polysaccharide were more widely prevalent in the patients who were successfully fighting the disease. Iland (17), on the other hand, could not find any antibodies to polysaccharide in serum from tuberculous human beings, but his test readings were made only at a twenty-four-hour period.

When highly purified protein and polysaccharide antigens became available, it seemed advisable to make a study of the simultaneous presence of antibodies to these antigens in the serum of animals and to study them both by the precipitin and the complement-fixation reactions. Moreover, an effort was made to determine the relationship between the presence of antibodies and the resistance of the host.

MATERIALS AND METHODS

Animals

All of the rabbits used in this study were white males which were purchased on the market except four of known resistance kindly given to us by Dr. Lurie. Rabbits T6=51, and T6=52 were from a strain known to be resistant to tuberculosis and AD5=33 and AD5=34 were from a less resistant strain (18).

Antiserums

Protein antiserum was obtained by taking blood from the ear veins of 7 rabbits which had been sensitized at five-day intervals by repeated intracutaneous injections of tuberculin protein fractions known to contain all or most of the *A* type of protein (*see* below). Ten-milligram doses were administered to each rabbit for eleven or twelve injections, at which time definite Arthus reactions appeared at the site of injection.

Antiserum was obtained also from 15 rabbits injected similarly ten to fifteen times, but with a suspension of live BCG organisms instead of purified protein. Each dose consisted of 0.25 mg. of the moist weight of the BCG organisms.

Other antiserums to PPD-S, the *C* type of tuberculoprotein, and bacillary protein (which had been frozen and saved from previous experiments) also were selected for study (19).

Antigens

Tuberculoprotein antigens: Details of the methods of isolation of tuberculoprotein antigens are given in earlier papers (20, 21). The *A*, *B*, *C*, and bacillary type of proteins contained 0.13, 18.8, 1.1, and 1.2 per cent of carbohydrate, respectively, determined by the anthrone method (22). PPD-S (23) also was used as an antigen for sensitization as well as in the precipitin and complement-fixation tests. This had 5.9 per cent carbohydrate.

Tuberculopolysaccharide antigens: Four highly purified tuberculin polysaccharides were used: two of them (Nos. 1 and 106), of low (7,000 to 9,000) molecular weight; and two (Nos. 85 and 92), very large molecules (100,000 molecular weight). Polysaccharide I was isolated from heated tuberculin filtrate of a human strain of tubercle bacilli (*DT*) by a chemical procedure described previously, in which basic lead acetate was used (24). It consisted of

arabinose and mannose and had only 0.27 per cent nitrogen or 1.68 per cent protein on the solid-weight basis. Polysaccharide No. 106 was prepared from the ultrafiltrate of a heated filtrate of a culture of human tubercle bacilli (*DT*). It was found by Professor M. Stacey to consist mainly of polyarabinose. Polysaccharides Nos. 85 and 92 were described previously as polyglucosans (13, 20), and were isolated from unheated tuberculins made from a bovine strain (No. 523) and a human strain (H37), respectively. The former contained 0.59 and the latter 0.31 per cent nitrogen.

All of these antigens dissolved readily in 0.9 per cent saline containing 0.01 per cent merthiolate, and were made to the following concentrations: 50, 10, 5, and 1 γ per 0.1 ml. These dilutions were found to span the titration range satisfactorily, sparing the serum for use with many antigens.

Tissue antigens: Attempts were made to recover tuberculoprotein and tuberculopolysaccharide directly from the tissues of tuberculous rabbits. The lung of a rabbit which showed extensive tuberculosis at autopsy was extracted with buffer, and then by autoclaving with water. The nitrogen content of the extracts was determined.

Immunologic Methods

Precipitin tests: Precipitin tests were carried out as follows: 0.1 ml. of serum was pipetted into each of five tubes (6 × 50 mm.), and then 0.1 ml. of each of the four concentrations of antigen was added, respectively, to four tubes. To the fifth tube 0.1 ml. of the buffer solution was added, as a control on the serum. To the sixth tube 0.1 ml. of the strongest concentration of antigen (50 γ) was added and then 0.1 ml. of the buffer, as a control on the antigen. All tubes were shaken, placed in the incubator for two hours, and kept at 4° C. overnight. The amount of precipitate was observed at the end of twenty-four hours and again in approximately four days. Frequently a much heavier precipitate occurred at the later reading. The amounts of precipitate found are shown in the tables as − (negative), · (trace), +, ++, +++, ++++, +++++, and ++++++.

Complement-fixation tests: Complement-fixation tests were carried out by the block titration method of T. N. Harris[1] on serum which had been inactivated at 56° C. for twenty minutes. In this method a series of dilutions of serum from 1:4 to 1:1,024 or more was titrated with a series of dilutions of antigen from 1:800 to 1:102,400, and all dilutions of serum and of antigen also were used as controls with buffered saline in order to detect any anti-complementary activity of either serum or antigen. Each tube contained 0.2 ml. of the respective serum dilution, 0.05 ml. of the respective antigen dilution, and 0.05 ml. of complement (diluted as indicated by previous titration). The tubes were incubated for thirty minutes in a water bath at 37° C., and 0.1 ml. of a mixture of equal parts of 4 per cent sheep cells and hemolysin (diluted as indicated by previous titration) was added to each tube. After thirty minutes in the water bath at 37° C., the tubes were placed in the cold room overnight before readings of the degree of hemolysis were made.

In this study some experiments were performed in which a second antigen was mixed with the PPD-S before the complement was added in order to determine the ability of this antigen to inhibit the complement-fixation reaction.

Chemical Analyses

Nitrogen analyses: All fractions were analyzed by the micro-Kjeldahl reaction and the concentrations of protein were calculated from these results.

Polysaccharide analyses: Analyses for polysaccharide in the antigens and tissue extracts were made by means of the tryptophane perchloric acid method (25) previously described, using levulose as a standard, and by the anthrone method described by Koehler (22), using a ten-minute heating period.

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[1] Dr. T. N. Harris and his associates kindly advised us on the technique for performing the complement-fixation test.

RESULTS

Precipitin Antibodies

Rabbits sensitized with tuberculoprotein: In table 1, data are shown on 7 rabbits, each of which has been sensitized by eleven intracutaneous injections of tuberculoprotein *A* (total of 110 mg.). All 7 rabbits had exhibited marked Arthus reactions, and their serums contained high concentrations of antibodies to tuberculoprotein *A*. The PPD-S, the *B* protein and, to a slightly less extent, the *C* protein were also good antigens in the precipitin reaction. The titers with these are not recorded in the table in order to save space. However, very little antibody to any of the three tuberculopolysaccharides, described under antigens, could be demonstrated in any of the serums obtained from these rabbits in several bleedings. No antibodies were found before the rabbits were sensitized.

Rabbits sensitized with BCG: In 15 rabbits sensitized by ten to fifteen weekly intracutaneous injections of a suspension of live BCG, a high content of antibodies to the three polysaccharides, as well as to all five types of protein antigens, was found. The three polysaccharide antigens appeared to be equal in their ability to precipitate the antiserums, but only one example is shown in table 1.

Two rabbits (Nos. 48 and 49), sensitized in the same way but with a suspension of heat-killed BCG, showed at least as high a concentration of all the antibodies as rabbits sensitized with live BCG organisms.

Distinction between precipitin antibodies to tuberculoprotein and tuberculopolysaccharide antigens: The character and range of the antigen-antibody precipitate with the protein antigens was very different from that with the polysaccharides. In the case of all of the protein antigens, the precipitates given with BCG antiserum usually were very heavy with 50 γ of protein per 0.1 ml. of serum and became progressively less until no precipitate occurred with 1 γ or less, depending upon the serum. When the tubes were tapped, a fine cloudlike suspension resulted.

On the other hand, with all three of the polysaccharide antigens there was frequently no precipitate with 50 γ, but with decreasing amounts of polysaccharide more and more precipitate occurred until maximum precipitation resulted with 1 γ or less of polysaccharide, and the end titer was as low as 0.001 γ of polysaccharide. Thus, the optimum precipitation point with polysaccharide always occurred at a much lower concentration than it did with the protein. Moreover, the protein antigen-antibody complex did not redissolve in antigen excess as did the polysaccharide antigen-antibody complex. The most distinctive characteristic of the polysaccharide antigen-antibody complex, however, was that it settled to a gelatinous pellet which dispersed to a granular suspension on tapping.

Complement-Fixing Antibodies

Relatively high complement-fixation titers were found in rabbits sensitized with BCG as is shown in tables 1, 2, and 3. PPD-S, *C*, and bacillary protein proved to be the best antigens for fixing complement with these antiserums (table 2). Similar results were found with an earlier series of rabbits by Dr. T. N.

TABLE I

PRECIPITIN AND COMPLEMENT FIXATION TITERS OF ANTISERUMS

Rabbit Number	Sensitizing Antigen	Tuberculoprotein A				Tuberculopolysaccharide No. 1				Complement-Fixation Titers with PPD-S
		50	10	5	1	50	10	5	1	
2	A	+++++	++++	++++	++	—	—	—	—	24
3	A	+++++	++++	++++	++	—	—	—	—	64
5	A	+++++	++++	++++	+++++	—	—	+	—	
7	A	++++	+++	+++	++	•	+	+		
11	A	+++++	++++	++++	++	•	+	•	•	24
14	A	+++++++	+++++	++++	+++	—	•	•	—	
16	A	+++++	++++	+++	++	—	—	—	—	0
6	Live BCG	++++	+++	+++	++	•	•	++	+++	
8	Live BCG	++++	+++	+++	++	•	++	++	++++	256
10	Live BCG	++++	++++	+++	++	—	—	+++	++++	512
17	Live BCG	++++	++++	+++	++	—	+++	++++	+++++	256
41	Live BCG	+++++++	++++	+++	+	•	++++	+++++	+++++++	768
42	Live BCG	++++	+++	++	++	—	+	++	+++	
44	Live BCG	+++++	++++	+++	+++	+	+++	++++	+++++	512
45	Live BCG	+++++	++++	+++	++	—	+	+++	++++	
46	Live BCG	+++++	+++++	+++++	+++	—	+	++	++++	384
47	Live BCG	+++++	++++	++++	+++	•	+	+++	+++++	192
43	Live BCG	+++++	++++	++	+	—	—	•	+	384
T6=51	Live BCG	++++++	+++++	+++++	+++	+	++++	+++++	++++++	256
T6=52	Live BCG	++++++	+++++	+++++	+++	+++	++++	+++++	++++++	192
AD5=33	Live BCG	+++++	+++	++	++	—	+	++	++	256
AD4=34	Live BCG	+++++	++++	++++	++	—	—	•	+++	256
48	Dead BCG	+++++	+++++	++++	+	+	++++++	++++++	++++++	>1,024
49	Dead BCG	+++++	+++++	++++	+	+	+++	+++++	++++++	
162	PPD-S	+++	+++	++	+	—	—	—	—	48
Gamma globulin	PPD-S	+++	+++	+++	•	+	—	—	—	6
Gamma globulin	Live tubercle bacilli	—	—	—		++	+	—		

TABLE 2

COMPLEMENT FIXATION BY CERTAIN ANTIGENS OF THE TUBERCLE BACILLUS IN BCG ANTISERUMS

Antigens	Relative Complement-Fixation Titers in the Same BCG Antiserum	Complement-Fixation Titers in Various BCG Antiserums
Tuberculoprotein A	12	0, 0, >32
Tuberculoprotein B	6	0
Tuberculoprotein C*	128, 192, 512	128, >128, >128
Bacillary tuberculoprotein	192	256, 256
PPD-S	512	256, 384, 256, 256, 192, 256, 768, 432, 512, 256, 384, 512
Tuberculopolysaccharide No. 1 and No. 106	0, 0	0, 0
Tuberculopolysaccharide No. 85		0, 0

* Three different preparations of C tuberculoprotein.

Harris (9). Tuberculoproteins *A* and *B* gave little or no fixation and the three tuberculopolysaccharides none at all, despite the fact that these antigens gave good precipitin reactions.

An explanation of the difference in the antigenic capacity of these antigens may come from earlier studies (26) which showed that the bacillary protein resembled the *C* more closely than it did the *A* type of protein in its amino-acid composition, electrophoretic mobility, and ultraviolet absorption. The PPD-S also contained a component similar to the *C* type of protein.

The complete lack of fixation by the tuberculopolysaccharide in BCG antiserum was interesting in view of the fact that such high precipitin titers had been obtained with this antigen. Since the optimum precipitation point was at such a low concentration of polysaccharide (1 to 0.001 γ per 0.1 ml. as mentioned above), block complement-fixation titrations were carried out with polysaccharide dilutions ranging from 1:800 to 1:800,000,000. At no dilution was there any evidence of fixation by the polysaccharide. Therefore, the polysaccharide could not be considered to be anticomplementary at any dilution. Similarly there was no evidence of anticomplementary action by the *A* or *B* types of tuberculoproteins.

In table 4 it may be noted that sensitization with the tuberculoproteins *A*, *C*, PPD-S, or bacillary protein, even to the extent where good Arthus reactions were obtained, did not elicit high complement-fixing antibodies in the serum of these sensitized animals in contrast to the serum of those sensitized with BCG.

Interference with an Antigen-Antibody Reaction

In addition to the fact that the tuberculopolysaccharide gave no complement fixation in BCG antiserum by itself, its presence interfered with the complement-fixing capacity of PPD-S when it was added to this system. Moreover, equal interference took place whether the PPD-S and polysaccharide were mixed

TABLE 3

COMPLEMENT FIXATION BY CERTAIN ANTIGENS AND INHIBITION IN BCG ANTISERUMS

Antigens	Complement-Fixation Titers	Ratio of Protein to Inhibiting Antigen
PPD-S	256	
PPD-S + tuberculopolysaccharide No. 1	48	1:7
PPD-S	256	
PPD-S + tuberculopolysaccharide No. 1	96	1:1
PPD-S + tuberculopolysaccharide No. 1	48	1:7
PPD-S	432	
PPD-S + tuberculopolysaccharide No. 1	54	1:8
PPD-S	384	
PPD-S + tuberculopolysaccharide No. 1	48	1:8
PPD-S + tuberculopolysaccharide No. 106	64	1:8
PPD-S + tuberculopolysaccharide (bovine) No. 85	256	1:8
PPD-S + tuberculonucleic acid	384	1:8
PPD-S	512	
PPD-S + glycogen	512	1:8
PPD-S + Winzler mucoprotein	512	1:8
PPD-S + tuberculoprotein A	192	1:8
PPD-S + tuberculoprotein B	96	1:8
PPD-S + tuberculoglycoprotein	32	1:8
PPD-S + tuberculopolysaccharide No. 1	64	1:8
PPD-S + tuberculopolysaccharide (human) No. 92	128	1:8
C	128	
C + tuberculopolysaccharide No. 1	12	1:2
Bacillary protein	192	
Bacillary protein + tuberculopolysaccharide No. 1	24	1:2
PPD-S	256	
PPD-S + isoniazid	256	1:1
PPD-S + tuberculopolysaccharide No. 1	96	1:1
PPD-S + tuberculopolysaccharide No. 1	48	1:7
PPD-S + tuberculopolysaccharide and isoniazid No. 1	64	1:1:1
Tuberculopolysaccharide No. 1	0	

together with complement before addition to the serum or when the polysaccharide was added to the serum before mixing with the PPD-S complement mixture. The extent of this inhibition by tuberculopolysaccharide of the complement fixation by PPD-S in BCG antiserum was considerable, as can be seen in table 3.

The complement fixation in BCG antiserum by the *C* type of protein and by the bacillary protein was also inhibited by the presence of tuberculopolysaccharide. In table 3 it is shown that the large molecular tuberculopolysaccharides

TABLE 4

COMPLEMENT FIXATION BY CERTAIN ANTIGENS AND INHIBITION IN OTHER THAN BCG ANTISERUMS

Sensitizing Tuberculoprotein Antigen	Tuberculoprotein and Tuberculopolysaccharide Antigens Used for Complement Fixation	Complement-Fixation Titers	Ratio of Protein to Inhibiting Agent
A protein	A protein	32	
	A protein + polysaccharide No. 1	24	1:7
C protein	C protein	96	
	C protein + polysaccharide No. 1	96	1:7
PPD-S	PPD-S	48	
	PPD-S + polysaccharide No. 1	24	1:7
Bacillary protein	Bacillary protein	6	
	Bacillary protein + polysaccharide No. 1	6	1:1

also caused some inhibition: slight with preparation No. 85 (from a bovine strain of tubercle bacilli) and more with preparation No. 92 from a human strain.

The specificity of the inhibition is emphasized in table 3 by the fact that other polysaccharide-containing substances, such as tuberculonucleic acid, glycogen, and the mucoprotein isolated by Winzler[4] (27), did not inhibit the complement-fixation reaction between PPD-S and BCG antiserums; neither did isoniazid. However, a small amount of inhibition to the complement fixation by PPD-S in BCG antiserum was caused by tuberculoprotein *A* (table 3) and, since this protein contained only a trace of polysaccharide (0.13 per cent), the tuberculoprotein may be considered responsible. The tuberculoprotein *B* and the tuberculoglycoprotein had stronger inhibiting powers but, since both contained considerable polysaccharide in their molecules, it would be impossible to consider these inhibitions to be due to protein.

In contrast to BCG antiserum, the experiments showed that relatively weak complement-fixing antibodies were found in serum of animals sensitized with *A*, *C*, or bacillary proteins. Furthermore, these low titers were effected very little, or not at all, by the addition of tuberculopolysaccharide to the system (table 4), indicating that the inhibition phenomenon was effective mainly on an antigen-antibody relationship that existed primarily in BCG antiserum.

Inhibition by tuberculopolysaccharide in the precipitin reaction, as well as in the complement-fixation test, was found in BCG antiserum. Much less precipitate, as well as a lower end titer, resulted when tuberculopolysaccharide was added to the system in such a way as not to change the original concentration relationship between serum and antigen. No similar effect was noticed when antiprotein instead of anti-BCG serum was used. Further evidence for the specificity of the reaction was furnished by the fact that addition of tuberculonucleic acid instead of tuberculopolysaccharide caused no inhibition.

Since, in the *in vitro* system, the tuberculopolysaccharide was capable of interfering so definitely with the complement-fixation reaction of serum of rabbits immunized with BCG, the *in vivo* role of this polysaccharide was studied.

[4] The mucoprotein was kindly furnished by Dr. R. Winzler.

Injections of tuberculopolysaccharide into rabbits Nos. 46 and 47, following infection, caused the disappearance of antibodies to the polysaccharide and a great depression of the complement-fixation titers (table 5), but considerable antibodies to the protein remained.

Infection alone was not the cause of disappearance of the antibodies to polysaccharide and the lowering of the complement-fixation titer. For example, in rabbits Nos. 53 and 57, with no treatment prior to infection, the complement-fixing titer rose slightly and precipitating antibodies to protein began to appear, as if the rabbits were endeavoring to resist the invasion of the bacilli.

Injections of tuberculoprotein intracutaneously at ten-day intervals (0.5 mg. per dose) also caused complete disappearance of the polysaccharide antibodies and a marked decrease in the complement-fixation titer, while at the same time the antibodies to protein became heavier. This occurred in rabbit No. 41 which had high antibody titers to tuberculoprotein and tuberculopolysaccharide antigens at the time of infection (table 5).

Relationship of the Presence of Antibodies to Resistance of the Animals

In rabbits sensitized with tuberculoproteins *A*, *C*, PPD-S, or bacillary protein, high precipitating antibodies to tuberculoprotein were obtained as shown in table 1 and also in earlier studies (9, 28). However, only low complement-fixing antibodies were found and practically no antibodies to tuberculopolysaccharide (table 1). In comprehensive previous studies (19, 28, 29), such animals have never shown any increased resistance to subsequent infection with virulent tubercle bacilli. In fact their resistance appeared to be lowered.

Nevertheless, the antiserum of rabbits which had been sensitized with PPD-S contained some antibacterial substance in the gamma globulin fraction which was able to inhibit the growth of tubercle bacilli in the test tube (30, 31) and to retard the development of tubercles on the chick membrane (31). One such PPD-S antiserum, as well as the gamma globulin fraction isolated from it, which had precipitating antibodies to the PPD-S showed a low complement-fixation titer (table 1). Complement-fixing antibodies have been found in general in gamma globulin (32, 33). The gamma globulin, isolated from the serum of a tuberculous rabbit which had had no treatment prior to infection, was found to contain antibodies to polysaccharide (table 1), which confirms reports by Cole and Favour (34). However, in spite of the fact that certain antibodies were present in the serum, none of these rabbits was immune when challenged (19).

In previous studies the large molecular polysaccharide was capable of acting as a true antigen by stimulating the production of antibodies to itself (13, 19), but animals in which these antibodies could be demonstrated were also not immune to infection with the tubercle bacillus (19).

Another experiment (28), in which the bacillary protein was repeatedly injected into guinea pigs, demonstrated that it also was not able to confer immunity on the animals, despite the fact that their cells in tissue cultures proved to possess a hypersensitivity similar to tuberculous cells.

On the other hand, it is well known that rabbits sensitized by repeated injec-

TABLE 5

ANTIBODY TITERS IN RABBITS IMMUNIZED WITH BCG AND AFTER INFECTION AND VARIOUS TREATMENTS

Rabbit Number	Treatment	Precipitin Titers to								Complement-Fixation Titers with PPD-S
		Tuberculoprotein A				Tuberculopolysaccharide No. 1				
		50	10	5	1	50	10	5	1	
57	Untreated before inoculation	—	—	—	—	—	—	—	—	32
	47 days after inoculation	+	•	•	•	•	•	•	+	48
53	Untreated before inoculation	—	•	•	•	—	—	—	—	16
	47 days after inoculation	+	+++	+++	—	—	—	—	—	24
44	At inoculation	+++	+++	+++	++	—	++++	++++	+++++	512
	49 days after inoculation	++++	+++	+++	++	—	+	+++	++++	256
43	At inoculation	+++++	++++	++	+	—	—	•	+	384
	49 days after inoculation	+++	+	+	+	—	—	—	—	128
46	Normal	—	•	•	•	—	•	—	•	0
	At inoculation	+++++	+++	+++	++	—	+	++	++++	384
	After 2 injections of polysaccharide No. 1	++++	+++	+++	++		—	—	—	192
	After 5 injections of polysaccharide No. 1	+++	++++	++++	+++	—	—	—	—	12
47	At inoculation	+++++	+++++	++++	+	—	—	+	+++	192
	After 5 injections of polysaccharide No. 1	+++	++++	++++	+++	—	—	—	—	16
41	At inoculation	++++++	+++++	+++	+	•	++++	+++++	++++++	768
	After 5 injections of protein A	++++++	++++++	++++++	++++++	—	—	—	—	128

tions of live BCG show a certain degree of immunity, and this was true in the experience of the present writers (19). In table 1, the precipitin and complement-fixing titers are given of many such rabbits after they had received ten to fifteen intracutaneous injections of a suspension of live or dead BCG. All of these rabbits showed high precipitating antibodies to tuberculoprotein and, in general, to tuberculopolysaccharide. Their complement-fixation titers also were very high. It was of interest that the 2 rabbits of known greater susceptibility (AD5=33 and AD5=34) made only scant antibodies to tuberculopolysaccharide on frequent tests during their course of injections. However, they were capable of producing as abundant precipitating antibodies to tuberculoprotein and as high complement-fixing antibodies to PPD-S as were the known resistant rabbits (T6=51 and T6=52). One market rabbit (No. 43) reacted to repeated injections of live BCG in the same manner as these 2 susceptible rabbits.

The Presence of Antigen in Tuberculous Tissues

If the presence of tuberculopolysaccharide is capable of exerting such a profound influence on the antibodies to itself, as well as on the complement-fixing antibodies, as has been demonstrated both *in vitro* and *in vivo*, its presence in the tissues could be of paramount importance. Although its presence in tuberculous lesions might be expected because of the large quantities of tubercle bacilli therein, it has never been demonstrated. Therefore, a further attempt was made to identify it in such lesions. An extract, made as described above under "*Tissue antigens*," was used as an antigen against an antiserum to BCG known to contain high concentrations of antibodies to both tuberculoprotein and tuberculopolysaccharide. From table 6 it may be seen that on two occasions a specific precipitate resulted. Since the precipitate had the granular appearance characteristic of polysaccharide antigen-antibody complexes, and since no precipitate resulted when antiserum containing only protein antibodies or normal serum was used, the conclusion may be drawn that the antigen present was specific tuberculopolysaccharide.

Similar experiments were performed in which extracts of tuberculous tissues made in other ways, the dialysates of tuberculous serum and of tuberculous urines as well as the residues after these dialyses, were used as antigens. All gave precipitates with anti-BCG serum, antiprotein serum, and with normal

TABLE 6

IDENTIFICATION OF TUBERCULO-ANTIGENS IN RABBIT TISSUES

Serum Used for Test	Extract of Tuberculous Lung	Dialysate of Tuberculous Serum	Saline
BCG antiserum	+, ++	++, ++	—
Protein A antiserum	—	++	—
Normal serum	— —	++	—
Saline	— —	—, —	

serum. Therefore, these precipitates could not be considered to contain specific antigen, but they require further study.

DISCUSSION

From the results of the present study and of previous studies, it is reasonable to believe that the simultaneous presence of antibodies to tuberculoprotein and to tuberculopolysaccharide, as well as complement-fixing antibodies, are necessary before a state of immunity to infection with the tubercle bacillus can exist, and that this delicate balance of antibodies would be especially important at the time of infection. Examples have been given in which antibodies only to the protein or antibodies only to the polysaccharide were present, and the experimental animals not only were not immune but fared even worse than untreated controls.

In animals sensitized with BCG, in which case it is recognized that some immunity generally does exist, all three types of antibodies always were present except in rabbits known to be susceptible to the disease. In the latter, the antibodies to polysaccharide were missing or deficient. In rabbits known to be resistant, immunization with BCG was capable of calling forth large amounts of all the antibodies, thus enhancing the native resistance of the animals, whereas the increment of immunity in susceptible rabbits was very slight. This phenomenon has been emphasized by Lurie (18). That certain rabbits purchased on the market succumb to infection more rapidly than the majority is a common experience. In the present study it has been shown that certain rabbits (like No. 43) are incapable of eliciting significant antibodies to the tuberculopolysaccharide when repeatedly injected with live BCG, just as were the rabbits of known genetic susceptibility. An experiment is underway to test further this hypothesis on such rabbits, and to determine whether passive transfer of the missing antibodies might be of value.

The present study shows that, in addition to the difficulty that certain rabbits have in producing antibodies to tuberculopolysaccharide, the tuberculopolysaccharide itself is capable of interfering with antigen-antibody relationships. Addition of this antigen caused *in vitro* inhibition to the complement-fixation reaction of BCG antiserum with PPD-S. This was not because it was anticomplementary in nature, for it fixed no complement by itself, but it specifically interfered with the complement-fixation reaction in antiserum produced by an antigen (BCG) which was capable of inducing immunity. On the other hand, it exerted little or no interference with the complement-fixing antibodies to antigens which were not capable of inducing immunity (table 4, *A* and *C* proteins, and PPD-S and bacillary proteins). Therefore, it is intriguing to wonder whether this polysaccharide may not be capable of interfering with immune reactions when sufficient of its own antibodies are not present to keep it from doing so. Such would be the case with the less resistant rabbits which are deficient in antibodies to the polysaccharide. The exact mechanism of this interference requires further study.

If such an hypothesis were true, the presence of free tuberculopolysaccharide

in the body would be detrimental. On searching for such free tuberculopolysaccharide, it was demonstrated to be present in tuberculous lung tissue. Moreover, the injection of excess tuberculopolysaccharide into immunized rabbits which possessed large quantities of antibodies caused the rapid disappearance of antibodies to itself, as well as a marked drop in the complement-fixing titer. Similar but less marked interference also was caused *in vitro* and *in vivo* by an excess of the tuberculoprotein of the *A* type. Thus the ability of the free tuberculopolysaccharide and, to a less extent, the tuberculoprotein to interfere with certain antigen-antibody relationships when their own antibodies are deficient may be one explanation why immunization procedures frequently fail to induce or maintain significantly enhanced resistance. Such interference also may explain the difficulty in demonstrating antibodies in human tuberculous serum, as has been suggested by Gerstl and associates (33). That specific polysaccharide, if in excess, can interfere with immune reactions in the body has been shown for another disease, pneumonia, by Felton and co-workers (35).

Since the tuberculopolysaccharide appeared to be such an important intruder in the body, efforts were made to determine its connection with the increase in the polysaccharide content of serum, which had been demonstrated to occur with the development of tuberculosis, especially when the prognosis was bad (25, 36, 37). Such polysaccharide was identified in an alpha globulin fraction of the serum (25) but, since it also was found in cancer and other diseases accompanied by tissue destruction, it was thought not to be specific for tuberculosis. The tryptophane-perchloric acid reagent was used to measure it (25). In the present study, the evidence indicated that this serum polysaccharide did not consist of tuberculopolysaccharide, for the latter gave no color reaction with the tryptophane-perchloric acid reagent.

SUMMARY

Antibodies to tuberculoproteins and tuberculopolysaccharides have been identified and distinguished by the precipitin reaction, using purified antigens from tuberculin. Complement-fixing antibodies were identified only by certain antigens, *C* type of protein, PPD-S, and bacillary protein.

Rabbits sensitized with any of the tuberculoprotein antigens had precipitating antibodies only to these proteins and low complement-fixation titers. Sensitization with the antigenic polysaccharide gave rise only to antibodies to polysaccharide. None of these rabbits was immune.

Sensitization with BCG, which usually results in immunity, induced precipitating antibodies to tuberculoprotein and tuberculopolysaccharide and high complement-fixing antibodies, except in rabbits of known genetic susceptibility. In the last-named animals, the antibodies to the polysaccharide were shown to be deficient.

Tuberculopolysaccharide and, to a less extent, tuberculoproteins (*A* and *B*) specifically interfered *in vitro* and *in vivo* with antigen-antibody reactions.

An hypothesis is given, on the basis of the experimental data, which considers the tuberculopolysaccharide, if free in the body, to be able to interfere with im-

mune reactions, and emphasizes the possible importance of a balanced antibody spectrum in immunity.

SUMARIO

La Importancia de los Anticuerpos a la Tuberculoproteína y los Polisacáridos en la Resistencia a la Tuberculosis. Impedimentos a los Anticuerpos por Dichos Antígenos

Los anticuerpos a las tuberculoproteínas y los tuberculopolisacáridos fueron identificados y diferenciados por la precipitinorreacción, usado antígenos purificados obtenidos de la tuberculina. Los anticuerpos fijadores del complemento fueron identificados únicamente por ciertos antígenos, la proteína de tipo C, el PPD-S y la proteína bacilar.

Los conejos sensibilizados con cualquiera de los antígenos tuberculoproteínicos no tenían más que anticuerpos precipitantes a dichas proteínas y mostraban títulos bajos de fijación del complemento. La sensibilización con el polisacárido antigénico no produjo más que anticuerpos al polisacárido. Ninguno de estos conejos quedó inmune.

La inmunización con BCG, que suele culminar en inmunidad, produjo anticuerpos precipitantes a la tuberculoproteína y al tuberculopolisacárido y títulos altos de antígenos fijadores del complemento, excepto en los conejos de conocida susceptibilidad genética, en los que los antígenos al polisacárido resultaron deficientes.

El tuberculopolisacárido y, en menor grado, las tuberculoproteínas (*A* y *B*) obstruyeron específicamente *in vitro* e *in vivo* las reacciones de antígeno-anticuerpo.

A base de los datos experimentales, ofrécese una hipótesis que considera al polisacárido como nocivo si está libre en el cuerpo, y recalca la posible importancia de un espectro equilibrado de anticuerpos en la inmunidad.

RESUME

L'importance des anticorps des tuberculo-protéines et des tuberculo-polysaccharides dans la résistance à la tuberculose. L'action inhibitrice de ces antigènes sur les anticorps

Les anticorps des tuberculo-protéines et des tuberculo-polysaccharides ont été identifiés et leurs caractères distinctifs ont été déterminés par la réaction de précipitation en utilisant des antigènes purifiés extraits de la tuberculine. Les anticorps fixant le complément ont été identifiés uniquement par certains antigènes, la protéine type C, PPD-S et protéine bacillaire.

Des lapins sensibilisés avec n'importe lequel de ces antigènes tuberculo-protéiques avaient des anticorps qui ne précipitaient qu'en présence de ces protéines et des titres de fixation du complément faibles. La sensibilisation au polysaccharide antigénique ne produisait que des anticorps au polysaccharide. Aucun de ces lapins n'était immun.

La sensibilisation au B.C.G., qui entraîne habituellement l'immunisation, provoquait l'apparition d'anticorps précipitant en présence de tuberculo-protéine et de tuberculo-polysaccharide et des anticorps ayant un taux de fixation du complément élevé, excepté chez les lapins ayant une sensibilité génétique reconnue et chez lesquels les anticorps au polysaccharide ont été reconnus déficients.

Les tuberculo-polysaccharides et, à un moindre degré, les tuberculo-protéines (*A* et *B*) intervenaient spécifiquement *in vitro* et *in vivo* dans les réactions antigène-anticorps.

En se fondant sur les données expérimentales, les auteurs formulent l'hypothèse de la nocivité du tuberculo-saccharide libre dans l'organisme et ils mettent l'accent sur l'importance possible du spectre anticorps équilibré dans l'immunité.

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SEROLOGIC INVESTIGATIONS OF MYCOBACTERIAL ANTIGENS[1]

I. The Specificities of Aqueous and Saline Extracts of BCG, *Mycobacterium Phlei*, and H37Rv

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INTRODUCTION

It is generally conceded that only polysaccharides are adsorbed to the surface of normal erythrocytes (1). Nevertheless, Gaby, Black, and Bondi (2) and Fisher (3) have demonstrated antigenic similarities and differences among the mycobacteria by sensitizing erythrocytes with tuberculins or phenolic extracts from various organisms and by testing the treated erythrocyte suspensions with specific antiserums. The type specificities of the mycobacteria are apparently associated with the protein components of the bacterial cell (4). Hence, in the studies cited, either type-specific polysaccharides, not previously recognized, were demonstrated by the erythrocyte sensitization or polysaccharide-protein complexes had been adsorbed with the protein portion of the complex directing the specificity. If the latter were the case, it would then become important in antigenic studies to obtain erythrocyte-sensitizing material from the test bacilli by procedures least likely to disrupt cellular complexes.

In the present investigation the antigenic relationships among erythrocyte-sensitizing antigens of Old Tuberculin (OT) and water and saline extracts of representative mycobacteria were examined. In order to avoid or minimize the loss of erythrocyte-sensitizing components, the bacillary masses were not defatted prior to extraction.

MATERIALS AND METHODS

Serum: The serum samples employed throughout this investigation were obtained from rabbits bled at weekly intervals following a single injection of 2.0 mg. of saline suspensions of BCG, *Mycobacterium phlei*, or *M. tuberculosis* var. *hominis* (H37Rv) or *bovis* (Ravenel). Prior to storage at −20° C., the serum was heat inactivated (56° C., for thirty minutes), adsorbed twice at room temperature with 0.1 volume of normal sheep cells (twenty minutes per adsorption) to remove heterophil antibody, and diluted 1:2 with phosphate buffered saline (pH 7.4). Aliquots of the serums were heated for an additional ten minutes at 70° C. before testing for the presence of heat-stable antibodies (5).

Hemagglutination and hemolytic tests: The technique for the hemagglutination test (6) consisted of mixing 2 drops of OT[4] (1:15)-sensitized sheep erythrocytes with equal volumes of doubling saline dilutions of the test serum, followed by incubation for thirty minutes at 37° C. After light centrifugation, the last serum dilution showing marked agglutination of cells was considered the end point of the titration. The hemolytic test was performed in

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precisely the same manner except that one drop of adsorbed guinea pig complement (1:3) was added to each tube in the titration (6).

Preparation of bacillary extracts: M. phlei and BCG were cultivated in Fernbach or Erlenmeyer flasks containing nutrient broth (Difco) to a depth of 2.5 cm. After ten to fourteen days' incubation at 37° C., the cells were harvested by centrifugation and the excess moisture was removed by pressing the bacillary mass between sheets of filter paper. Two and one-half grams of cells were mixed with 12.5 ml. of water or phosphate buffered saline (pH 7.4) and were incubated for two hours in a 37° C. water bath with frequent agitation. The supernatant fluid was removed after centrifugation at 3,000 r.p.m. for one hour in the cold, and filtered through a Selas filter. The aqueous extracts were made isotonic by the addition of dry sodium chloride.

A strain of *M. tuberculosis* of human origin (H37Rv) was cultivated in Sauton's medium for three weeks and the cells were recovered by careful decantation. The bacilli were washed free of medium with cold saline and were used in the preparation of an aqueous extract as outlined above. This preliminary washing was found necessary since the glycerol in the residual medium was sufficient to affect adversely the sheep erythrocytes during sensitization.

Sensitization of sheep erythrocytes with crude bacillary extracts: Four milliliters of the appropriate bacillary extract were mixed with 0.1 ml. of packed sheep cells and were incubated for two hours in a 37° C. water bath. The sensitized cells were washed three times with buffered saline and employed in the serologic tests as a 0.5 per cent suspension. The details of the testing procedures have been described above.

RESULTS

Serologic Activity of Erythrocytes Sensitized with Aqueous or Saline Extracts of BCG and M. Phlei

Other reports from this laboratory have described the development of antibody patterns in rabbits infected or immunized with mycobacteria (5, 7). In order to compare the antigenic specificity and sensitivity of erythrocytes treated with crude aqueous or saline extracts of BCG and *M. phlei*, serums procured at weekly intervals were tested for hemolysins and hemagglutinins as outlined above.

Representative titrations, recorded in table 1, indicated that extraction of bacilli with water or saline resulted in the successful removal of erythrocyte-sensitizing material and that extract-treated cells exhibited serologic specificities not seen with OT-sensitized cells. It may be noted, for example, that sheep cells sensitized with the saline extract from *M. phlei* did not permit unheated serum hemagglutinins or either heat-stable antibody to be demonstrated in any of the serums tested. Moreover, serums from 14 animals, not recorded here, gave similar results.

Of the four series recorded, only those serums from rabbits injected with *M. phlei* or BCG showed heated serum antibodies when tested with the other extract antigens. Occasionally, however, these antibodies had been detected in serum samples from infected rabbits, thus suggesting that the general lack of demonstration might be attributed to a low concentration, rather than absence, of antigen in the extracts.

Generally, the anti-*M. phlei* and anti-BCG serums reacted in higher dilutions with the extract-treated cell preparations than did the serums from infected animals. In addition, the aqueous extracts of both organisms were serologically

TABLE 1

ERYTHROCYTE-SENSITIZING CAPACITIES OF WATER AND SALINE EXTRACTS OF BCG AND M. PHLEI

Erythrocytes Sensitized with:	Weeks Post Injection	Anti-*M. phlei*				Anti-BCG				Anti-H37Rv				Anti-Ravenel			
		HL*	HA	Δ HL	Δ HA	HL	HA	Δ HL	Δ HA	HL	HA	Δ HL	Δ HA	HL	HA	Δ HL	Δ HA
OT(1:15)	0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	1	1,024	128	—	—	512	128	—	—	128	16	—	—	64	8	—	—
	2	2,048	512	—	—	512	512	—	—	256	16	—	—	512	64	—	—
	3	512	128	2	2	1,024	128	4	4	256	64	8	8	512	128	2	—
	4	512	128	16	8	256	64	4	2	512	128	16	8	1,024	128	128	—
	5	256	64	32	8	256	4	8	4	256	128	16	16	1,024	256	64	8
BCG extract (water)	0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	1	4,096	1,024	4	—	2,048	512	4	2	256	64	—	—	128	16	—	—
	2	8,192	1,024	4	4	2,048	1,024	4	2	128	16	—	—	512	64	—	—
	3	2,048	1,024	16	8	4,096	1,024	8	8	512	32	—	—	1,024	64	—	—
	4	2,048	256	32	16	2,048	512	32	16	256	32	—	—	1,024	128	—	—
	5	1,024	256	64	32	2,048	256	32	16	256	32	—	—	512	128	—	—
M. phlei extract (water)	0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	1	4,096	256	—	—	256	32	—	—	16	4	—	—	16	8	—	—
	2	8,192	512	—	—	512	128	—	—	8	4	—	—	32	16	—	—
	3	1,024	256	4	—	256	64	—	—	64	2	—	—	128	4	—	—
	4	512	128	16	16	256	32	8	—	32	4	—	—	128	32	—	—
	5	512	64	16	16	128	32	8	—	32	8	—	—	128	32	—	—
BCG extract (saline)	0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	1	512	256	—	—	512	64	—	—	16	8	—	—	16	2	—	—
	2	2,048	256	—	—	512	128	—	—	16	4	—	—	64	4	—	—
	3	1,024	512	2	—	512	64	2	2	32	16	—	—	128	4	—	—
	4	512	256	8	—	256	32	4	2	64	16	—	—	128	32	—	—
	5	512	128	16	—	128	16	16	2	128	16	—	—	512	64	—	—
M. phlei extract (saline)	0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	1	128	—	—	—	32	—	—	—	4	—	—	—	2	—	—	—
	2	256	—	—	—	256	—	—	—	4	—	—	—	8	—	—	—
	3	128	—	—	—	128	—	—	—	16	—	—	—	8	—	—	—
	4	128	—	—	—	64	—	—	—	8	—	—	—	64	—	—	—
	5	32	—	—	—	16	—	—	—	8	—	—	—	32	—	—	—

* HL denotes hemolysin; HA, hemagglutinin; Δ, heat-stable antibodies.

more reactive than the corresponding saline extracts as measured by antibody demonstration. For these reasons the aqueous extracts were used for the following experiments.

Inhibition tests: The results recorded in table 1 showed specificities that could be investigated most successfully by inhibition tests. Accordingly, a mixture of one drop of 1:15 OT or undiluted aqueous extract and 2 drops of antiserum (diluted 1:8), was incubated in a 37° C. water bath for thirty minutes. Following the addition of 2 drops of the appropriate sensitized-cell suspension, the mixture was reincubated for thirty minutes. Inhibition was indicated by the absence of hemagglutination.

Inhibition of hemolysis was measured in a similar manner except that one drop of a 1:3 saline dilution of adsorbed complement was included. In order to ascertain the diluting effects of the extracts, controls were prepared as above except that one drop of saline was substituted for the aqueous extracts. The serums employed in these tests were those described in the previous experiment.

From the data tabulated in table 2, it may be seen that all the hemolytic controls were positive. The failure of certain serums to produce agglutination was attributed to dilution by the saline and the variable recovery of the agglutinogen from different preparations of the extracts, which, at present, is unexplained.

Old Tuberculin, 1:15, was completely effective in preventing all serums from reacting with OT-sensitized cells. It also inhibited hemolysis and hemagglutination of BCG extract-sensitized cells by anti-H37Rv and anti-Ravenel serums, but did not affect the demonstrations with anti-*M. phlei* and anti-BCG serums. Heterologous hemolysis was not demonstrated with *M. phlei* extract–treated cells in the presence of OT, and it would appear that the early homologous serum was also neutralized. Erratic controls did not permit the attaching of significance to the inhibition of hemagglutination by OT or by the bacillary extracts with any of the serums and this antigen suspension.

BCG-water extract inhibited reactions in all systems except with OT cells and antiserums from infected animals, while the *M. phlei* extract effectively blocked reactions only between serums from tuberculous rabbits and cells sensitized with BCG or *M. phlei* extracts.

To further examine the inhibiting properties of these extracts, 20 specimens of serum were tested with no regard to their absolute titers. Since previous studies had shown some of these to be devoid of agglutinins, however, only hemolytic tests were performed.

An aqueous extract of *M. tuberculosis* H37Rv was included in this experiment. A preliminary study had indicated that erythrocytes sensitized with this extract reacted similarly to the standard OT-treated erythrocytes when tested with several specimens of antiserum.

In general the results (table 3) verify the previous findings and emphasize the resemblance between the extract from the H37Rv strain and the standard OT, as measured by inhibiting properties. The failure of certain serums to react as anticipated can be explained most readily by the crudeness of the extracts.

Cross-adsorption studies: The inhibition reactions in the previous experiments suggested that material with limited serologic specificity was present in the

TABLE 2

HOMOLOGOUS AND HETEROLOGOUS INHIBITION BY OT AND AQUEOUS EXTRACTS OF BCG AND M. PHLEI

Erythrocytes Sensitized with:	Blocking Extract	Demonstration	Antiserums: *M. Phlei* Weeks post Injection 1	2	3	4	5	*BCG* 1	2	3	4	5	*H37Rv* 1	2	3	4	5	*Ravenel* 1	2	3	4	5
OT (1:15)	Saline	HL	+[a]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		HA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
BCG-water		HL	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		HA	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+
M. phlei-water		HL	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		HA	+	+	−	−	−	+	+	−	+	−	−	−	−	+	+	−	+	+	+	+
OT (1:15)	OT (1:15)	HL	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		HA	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
BCG-water		HL	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−
		HA	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−
M. phlei-water		HL	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		HA	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
OT (1:15)	BCG (water)	HL	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+
		HA	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+
BCG-water		HL	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		HA	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
M. phlei-water		HL	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		HA	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
OT (1:15)	*M. phlei* (water)	HL	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		HA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+
BCG-water		HL	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−
		HA	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−
M. phlei-water		HL	−	+	+	−	−	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−
		HA	+	+	+	+	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−

[a] + denotes hemolysis or hemagglutination.

aqueous extracts of BCG and *M. phlei*. This was verified by adsorption of the various antiserums with sheep cells sensitized with homologous and heterologous extracts. The adsorptions were performed by mixing an equal quantity of sensitized cells with each of the test antiserums, followed by incubation at room temperature for ten minutes. The adsorptions were repeated until the serums no longer reacted with the adsorbing antigen.

TABLE 3

HOMOLOGOUS AND HETEROLOGOUS INHIBITION OF HEMOLYSIS BY OT (1:15) AND AQUEOUS EXTRACTS OF H37Rv, BCG, AND M. PHLEI

Erythrocytes Sensitized with:	Blocking Extract	Antiserums																	
		M. phlei			BCG					H37Rv				Ravenel					
OT (1:15)	Saline	+°	+	+	+	+	+	+	+†	+	+	+	+	+	+	+	+	+†	+†
H37Rv-water		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
BCG-water		+	−	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+
M. phlei-water		+	+	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+
OT (1:15)	OT (1:15)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
H37Rv-water		−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−
BCG-water		−	−	−	+	+	−	+	+	−	−	−	−	−	−	−	−	−	−
M. phlei-water		−	−	+	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−
OT (1:15)	H37Rv Extract (water)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
H37Rv-water		−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
BCG-water		−	−	−	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−
M. phlei-water		−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
OT (1:15)	BCG extract (water)	−	−	−	−	−	+	−	−	+	+	+	+	+	+	+	+	+	+
H37Rv-water		−	−	−	−	−	+	−	−	+	+	+	+	+	+	+	+	+	+
BCG-water		−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
M. phlei-water		−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
OT (1:15)	*M. phlei* extract (water)	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+
H37Rv-water		−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+
BCG-water		−	−	−	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−
M. phlei-water		−	−	+	−	−	−	+	+	−	−	−	−	+	−	+	−	−	−

° + denotes hemolysis.

† Indicates animals immunized with heat-killed organisms.

A comparison of the results recorded in table 4 with those in tables 2 and 3 demonstrates the agreement between the adsorption and inhibition studies. The OT-sensitized cells removed all antibody except that detected by the homologous systems with *M. phlei* and BCG extract-sensitized cells. It can be seen that the reduction in the titer of the *M. phlei*–anti-*M. phlei* system is much greater than that observed in the BCG–anti-BCG system. The *M. phlei*-sensitized cells removed antibody from the anti-Ravenel serum so that it no longer reacted with BCG-sensitized cells. However, the antibodies responsible for the OT–anti-Ravenel reaction were only partially removed. The hemolytic antibody in anti-*M. phlei* serum, as measured with BCG extract-sensitized cells, was reduced but not eliminated, and the agglutinating antibody was apparently unaffected.

The BCG-sensitized cells removed all antibody except that measured with the OT-cell–anti-Ravenel system.

DISCUSSION

The above data clearly indicate that erythrocyte-sensitizing substances may be extracted with water or saline at 37°C. from untreated bacillary masses of myco-

TABLE 4

ADSORPTION OF ANTISERUMS BY CELLS SENSITIZED WITH OT (1:15) AND AQUEOUS EXTRACTS OF BCG AND M. PHLEI

Antiserums	Adsorbing Cells	Titers After Adsorption					
		Test Antigens					
		OT (1:15)		*M. Phlei* Extract		BCG Extract	
		HL	HA	HL	HA	HL	HA
Ravenel	—	256	64	16	8	32	16
M. phlei	—	1,024	128	1,024	16	2,048	16
BCG	—	1,024	512	4,096	2	4,096	256
Ravenel	OT (1:15)	—	—	—	—	—	—
M. phlei		—	—	8	4	—	—
BCG		—	—	—	—	512	128
Ravenel	*M. phlei*	32	4	—	—	—	—
M. phlei	Extract	256	64	—	—	128	32
BCG	(water)	256	64	—	—	2,048	128
Ravenel	BCG	8	4	—	—	—	—
M. phlei	Extract	—	—	—	—	—	—
BCG	(water)	—	—	—	—	—	—

bacteria. Moreover, the extracts exhibited specificities comparable with those of antigens recovered by more involved procedures. The apparent decrease in the number of antigens obtained by extracting *M. phlei* with saline as compared to the number of antigens in the water extract presents an interesting area for further investigation. At present, studies are in progress to determine if the differences are of a quantitative or qualitative nature and to determine the responsible factors.

Heated serum antibodies were demonstrated with OT-sensitized cells in some antiserum to all of the mycobacteria. The only demonstration with extract-sensitized cells, however, was in antiserums to *M. phlei* and BCG. As has been mentioned, this limited demonstration cannot be generalized to include all instances because the lack of demonstration with some serum may have been due to a low concentration of antigen. However, in the anti-BCG and anti-*M. phlei* serums the heated serum antibody demonstrations with BCG-water-extract-sensitized cells equals or exceeds those observed with OT cells. The failure to detect heated serum antibodies in anti-H37Rv and anti-Ravenel serums tested with extract-treated erythrocytes suggests that the specific antigens responsible for the heat-stable antibody demonstrations differ in the extract antigens and OT.

The results of the various titration, inhibition, and adsorption tests support the view that the mycobacteria contain common antigenic substances or demonstrate the so-called "antigenic overlap" (3). Furthermore, the data provide confirmatory evidence for the close antigenic relationship between the human and bovine strains of tubercle bacilli while *M. phlei* appears to be antigenically

more distinct. As might be expected, BCG occupies an intermediate position, although it is readily differentiated from the virulent strains by the inhibition and adsorption tests.

The extraction of antigens from bacillary masses by the mild methods described here has the advantage of leaving the cells intact. The possibility is presented, therefore, that repeated extraction of the same bacillary mass may provide a means of investigating different antigenic substances, depending on their solubility, quantity, and spatial position in the bacterial cell. The report of such an investigation is the subject of the following paper.

SUMMARY

Erythrocyte-sensitizing antigens can be obtained readily from masses of non-defatted mycobacteria by extraction with water or saline at 37°C. These antigens exhibit specificities more limited than those demonstrated with Old Tuberculin.

SUMARIO

Investigaciones Serológicas de los Antígenos Micobacterianos

Pueden obtenerse fácilmente antígenos sensibilizadores de los eritrocitos mediante la extracción con agua o solución salina a 37° C. de masas de micobacterias sin desgrasar. Estos antígenos manifestan especificidades más limitadas que las reveladas por la tuberculina antigua.

RESUME

Investigations sérologiques des antigènes mycobactériens

Les antigènes sensibilisateurs de l'érythrocyte peuvent être facilement obtenus, à partir de masses de mycobactéries non dégraissées, par extraction à l'eau ou solution physiologique à 37° C. Ces antigènes disposent de qualités spécifiques plus limitées que celles démontrées par la tuberculine de Koch (OT).

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SEROLOGIC INVESTIGATIONS OF MYCOBACTERIAL ANTIGENS[1,2]

II. Specificities of Antigens Obtained by Successive Aqueous Extraction of BCG and *Mycobacterium Phlei*

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INTRODUCTION

It is well recognized that antigens associated with bacteria do not necessarily exist in a dispersed state throughout the organism, but may be found in layers or strata. Notable examples of antigenic stratification occur with pneumococci, *Streptococci*, and *Salmonella*. Meynell has reported that the superficial antigens of a strain of tubercle bacillus of human origin are apparently polysaccharide while those more deeply situated are protein (1).

As it has been established (2) that simple water or saline extraction removes erythrocyte-sensitizing substances from non-defatted mycobacteria, it was decided to determine if repeated extraction of the same bacillary mass would result in antigens with different specificities. These differences might then be correlated with the relative solubilities and position of the antigens in the bacterial cell.

MATERIALS AND METHODS

The details of the extracting procedures and the preparation of reagents were essentially those described in the preceding paper (2). Although buffered saline was found effective as an extracting agent, larger yields of antigenic substances were obtained with distilled water. Therefore all extracts reported in this study were prepared with water and made isotonic with dry sodium chloride as was done in the previous experiments (2).

Bacillary masses of BCG and *Mycobacterium phlei* were extracted by suspending the organisms in water, followed by incubation, centrifugation, and filtration. The same bacillary masses were then subjected repeatedly to these procedures. The successive extracts were employed to sensitize normal sheep erythrocytes which were tested with homologous and heterologous antiserums.

EXPERIMENTAL RESULTS

Specificities of Successive Aqueous Extracts of BCG and Mycobacterium Phlei

From the results tabulated in table 1, it may be seen that the erythrocyte-sensitizing lytic antigens present in the later BCG extracts retain their specificity for the homologous serum while the antigens reacting with heterologous serums apparently are removed or reduced to a minimum after two extractions. This is not the case with the lytic antigens from successive extractions of *M. phlei*, in which the cross-reacting antigens are still present after several extractions.

The BCG agglutinogen disappears disproportionately upon successive extrac-

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TABLE 1

SPECIFICITY OF SUCCESSIVE AQUEOUS EXTRACTS OF BCG AND M. PHLEI

Erythrocyte-Sensitizing Extract	Antiserums							
	BCG		*M. Phlei*		H37Rv		Ravenel	
	HL*	HA†	HL	HA	HL	HA	HL	HA
BCG I	4,096‡	512	128	—	4	—	16	—
BCG II	2,048	128	128	—	8	4	32	32
BCG III	1,024	—	16	—	—	—	—	—
BCG IV	128	—	—	—	—	—	—	—
BCG V	256	—	4	—	—	—	—	—
M. phlei I	512	—	256	—	4	—	16	—
M. phlei II	512	—	256	—	16	—	32	—
M. phlei III	512	—	128	—	16	—	32	—
M. phlei IV	512	—	128	—	16	—	32	—
M. phlei V	128	—	16	—	—	—	4	—

* Hemolytic titer

† Hemagglutination titer

‡ Titer expressed as the reciprocal of the dilution

tion, emphasizing its independence from the lytic antigen. No *M. phlei* agglutinogen was demonstrable in these extracts.

While it is tempting to speculate further on the demonstrations of the agglutinogens in the various extracts, sufficient titrations of the type just described have not been conducted to permit predicting in which extract the agglutinogen will be present or absent. It should be remembered that, while these extracting procedures are revealing, the resultant extracts are crude preparations.

The Effects of Concentration on the Specificities of Aqueous Extracts of BCG and M. Phlei

Since the lytic antigen from later extracts of BCG apparently retained its specificity when tested with homologous serum, an attempt was made to further define this antigen.

BCG cells were extracted repeatedly and erythrocytes were sensitized with each extract. The last extract which either failed to sensitize cells or to which test antiserums reacted minimally was then concentrated eight times by vacuum desiccation at room temperature, made isotonic, and again used to sensitize sheep erythrocytes. For purposes of comparison, the sixth extract from *M. phlei* was treated identically, although it had been observed (table 1) that the antigen responsible for the reaction with anti-*M. phlei* serum disappeared more rapidly than the antigen which reacted with anti-BCG serum.

Erythrocytes sensitized with the concentrated twelfth BCG extract reacted with homologous serums to titers similar to those observed with cells treated with the first extract, while heterologous serums reacted minimally or not at all (table 2).

Erythrocytes sensitized with the concentrated sixth extract from *M. phlei* failed to give similar results, since the heterologous antigens were obviously

TABLE 2

THE EFFECT OF CONCENTRATION ON THE SPECIFICITY OF AQUEOUS EXTRACTS OF BCG AND M. PHLEI

Erythrocyte-Sensitizing Extract	Hemolytic Titers of Antiserums to:							
	BCG		*M. phlei*		H37Rv		Ravenel	
	A	B	A	B	A	B	A	B
BCG I	4,096	4,096	2,048	1,024	256	32	32	1,024
BCG XII	16	—	—	—	—	—	—	—
BCG XII (8 ×)	2,048	128	±8	±8	±2	±4	—	—
M. phlei I	512	4,096	1,024	512	32	16	16	32
M. phlei VI	512	64	32	16	—	4	—	—
M. phlei VI (8 ×)	1,024	1,024	1,024	256	32	32	16	4

still present. This is in keeping with the findings recorded in table 1, in which it may be observed that cells sensitized with subsequent extractions of *M. phlei* resulted in a progressive decrease of antigen detectable by both homologous and heterologous serums. Further extractions of *M. phlei* cells did not disclose any change in the range of specificity.

Influence of Dilution and Concentration on Erythrocyte-Sensitizing Capacities of Aqueous Extracts of BCG

The detection of antibody with sensitized erythrocytes implies a minimum quantity of antigen on the erythrocyte surface. Conversely, there is a maximum quantity of any one antigen which may be adsorbed (3). Although this is in part a function of the species of erythrocyte being tested, other factors, such as adsorbed but serologically nonreactive substances, are of importance.

In order to examine the influence of dilution and concentration on erythrocyte sensitization, the first BCG extract was either concentrated eight times, diluted 1:5, 1:10, and 1:15, or used as the undiluted extract. Suspensions of sheep cells sensitized with each of these preparations were tested with homologous and heterologous serums.

From the data (table 3) it appears that sufficient homologous antigen was

TABLE 3

ERYTHROCYTE-SENSITIZING CAPACITY OF DILUTED AND (8 ×) CONCENTRATED AQUEOUS EXTRACTS OF BCG

Erythrocyte Sensitizing Extract	Antiserums							
	BCG		BCG		*M. phlei*		H37Rv	
	HL	HA	HL	HA	HL	HA	HL	HA
BCG I	4,096	256	256	64	128	—	32	8
BCG I (8 ×)	8,192	512	1,024	256	2,048	—	256	64
BCG I 1:5	1,024	128	128	8	—	—	—	—
BCG I 1:10	512	64	64	—	—	—	—	—
BCG I 1:15	512	—	64	—	—	—	—	—

present in the undiluted extract to result in maximal or nearly maximal erythrocyte sensitization, as concentration (eight times) of the extract had little influence on the anti-BCG titer. The demonstration of the cross-reacting antigens, however, was enhanced by the concentration procedure.

The effect of diluting the extract prior to sensitization of erythrocytes indicated the specificity of the test antigen. Anti-BCG serum reacted with erythrocytes treated with the 1:15 dilution of extract while neither of the heterologous serums reacted with cells sensitized with the 1:10 or 1:5 dilutions. Thus the BCG extract just described apparently contained both specific and cross-reacting antigens.

DISCUSSION

The results recorded in the present report indicate that BCG may have an antigenic structure similar to that of the strain of tubercle bacillus of human origin described by Meynell (1). Therefore the cross-reactions with the initial extracts could be explained in terms of the superficial polysaccharide antigens which were eliminated upon continued extraction. The strain-specific antigens, less superficially located in the bacterial cell, could then predominate in the later extracts.

On the other hand, this stratification of antigens seems to be lacking in *M. phlei* since antigens for both homologous and heterologous serums were generally demonstrable.

The question of the adsorptive capacity of the normal erythrocyte has a direct bearing on the problem at hand. There is increasing evidence to indicate that adsorption of antigenic material by erythrocytes is quite selective and depends on the presence of particular erythrocyte-receptor sites. Landy (3) reported that the quantity of a given antigen that could be adsorbed by an erythrocyte was limited and minimized the probability of steric hindrance as the limiting factor by adsorbing purified antigens from several different bacteria to the same erythrocytes. The results obtained with the diluted and concentrated first aqueous BCG extract suggest that the homologous or species specific antigens may be adsorbed to erythrocyte receptor sites different from those required by the cross-reacting antigens.

Since it is generally recognized that the protein antigens of the mycobacteria represent the most strain-specific material in the organism (4), it becomes important to consider whether protein antigens could be adsorbed by normal erythrocytes. Meynell (1) has suggested that the terms "protein" and "polysaccharide" should be used with caution, remembering the complexity of the chemical components in the mycobacteria. It is probable that, under the mild extracting conditions employed here, chemically pure components would not be isolated and the "protein" would be in association with polysaccharide material. The latter could provide the means of adsorption to the erythrocyte. The alternative to this possibility is the existence of a strain specific mycobacterial polysaccharide for which there is little, if any, precedence in the literature on tuberculosis (5).

It is suggested that the mild extraction procedure described here and in the previous paper (2), combined with the selective adsorptive capacity of erythrocytes, offers a convenient means for studying the antigens of the mycobacteria. Perhaps further investigation with these techniques will reveal fundamental differences in the position of the antigens found in saprophytic, attenuated, and virulent strains of mycobacteria. Similar observations among other bacteria have been of importance in selecting the proper antigen for use in diagnosis or immunization.

SUMMARY

Specific erythrocyte-sensitizing antigens were obtained by repeated extraction of BCG cells. The arrangement or solubility of these antigens in BCG seemingly differs from those in *M. phlei*. The technique of repeated extraction of the same bacillary mass offers a method of investigating various mycobacterial antigen-antibody systems.

SUMARIO

Investigaciones Serológicas de los Antígenos Micobacterianos. II. Especificidades de los Antígenos Obtenidos por Extracción Acuosa Sucesiva del BCG y del Mycobacterium phlei

Obtuviéronse antígenos específicos sensibilizadores de los eritrocitos mediante la extracción repetida de células de BCG. La disposición o solubilidad de estos antígenos en BCG discrepa aparentemente de la observada en *M. phlei*. La técnica de la extracción repetida de la misma masa bacilar ofrece un método para investigar varios sistemas micobacterianos y de antígeno-anticuerpo.

RESUME

Investigations sérologiques des antigènes mycobactériens. II. Qualités spécifiques des antigènes obtenus à partir de BCG et de M. phlei par extraction aqueuse répétée

Des antigènes spécifiques, sensibilisateurs de l'érythrocyte, ont été obtenus par l'extraction répétée à partir de cellules de BCG.

La disposition ou la solubilité de ces antigènes dans le BCG semble différer de celles observées pour le *M. phlei*. La technique de l'extraction répétée à partir d'une même masse bacillaire offre une méthode permettant l'investigation de divers systèmes mycobactériens et antigènes-anticorps.

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(3) Landy, M.: On hemagglutination procedures utilizing isolated polysaccharide and protein antigens, Am. J. Pub. Health, 1954, *44*, 1059.

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Notes

THE DEVELOPMENT OF A RAPID MICROCULTURE TECHNIQUE FOR PRIMARY ISOLATION OF MYCOBACTERIUM TUBERCULOSIS[1]

The cultural methods which have been developed for primary isolation of tubercle bacilli, although time-consuming, are quite reliable. Improved media are available which support the growth of small numbers of tubercle bacilli, and differentiation can usually be made with respect to pathogenic and saprophytic acid-fast bacilli. Despite the improvements in media there has been little reduction in the time (three to eight weeks) required for culture of the organisms, and it appears unlikely that further changes in the composition of media will appreciably reduce the relatively long incubation period. However, if a satisfactory method were available for the detection of early colonial growth, cultural demonstration of *M. tuberculosis* could be accomplished in a few days.

Several microculture techniques for the primary isolation of tubercle bacilli have been described,[2-6] but these techniques have proved deficient in lack of sensitivity or have been too laborious for routine use. The present paper embodies a description and the preliminary evaluation of a simplified method of microculture for the primary isolation of *M. tuberculosis*, which allows the use of alkali-treated, concentrated specimens. The procedure for microculture and the method of evaluation were as follows:

Sputum specimens to which an equal quantity of 4 per cent sodium hydroxide had been added were placed in a bottle shaker and subjected to approximately 250 to 300 oscillations per minute for fifteen minutes. The specimens were then transferred to screw-cap test tubes and centrifuged at 3,000 r.p.m. for fifteen minutes. The supernatant was discarded. One drop of a 0.04 per cent solution of phenol red was added to each sediment, and normal hydrochloric acid was carefully added by means of a capillary pipette until a faint pink color was attained. Each neutralized specimen was buffered by the addition of 0.1 ml. of sterile 0.1 M phosphate buffer pH 7.0, to each 2 ml. of sediment. Five milliliters of sterile 1 per cent Trypticase® (B.B.L.) broth were then added to each neutralized sediment which was subsequently resuspended. The specimens were again centrifuged at 3,000 r.p.m. for fifteen minutes, after which the supernatants were discarded.

From each specimen, 0.1 ml. of the sediment was transferred by means of a pipette and was spread on the surface of each of five sterile, narrow split-glass slides half the usual width, which were contained in two labeled Petri plates. The Petri plates had been modified by the insertion of a layer of No. 3 Whatman filter paper on the bottom of each plate and by the use of a cover fashioned from identical filter paper to hasten drying. Prior to use, sterilization of the modified plates containing the narrow slides was accomplished by heating in an autoclave equipped with vacuum or in a hot air oven. The plates containing the inoculated slides were placed in a shallow cardboard tray, covered with wrapping paper and allowed to remain at room temperature for forty-five to ninety minutes, at which time the sediment had usually dried. After drying, two of the slides were transferred by means of forceps, which had been dipped

[1] Presented in part at the meeting of The Society of American Bacteriologists, held in New York, New York, 1955.

[2] Pryce, D. M.: J. Path. & Bact., 1941, *53*, 327.

[3] Rosenberg, K. S.: Lancet, 1943, *1*, 615.

[4] Berry, J. W., and Lowry, H.: Am. J. Clin. Path., 1950, *20*, 1037.

[5] Hesselberg, I., and Oeding, P.: Acta tuberc. Scandinav., 1952, *26–27*, 217.

[6] Hoyt, A., *et al.*: Am. Rev. Tubere., 1954, *70*, 916.

in alcohol and flamed, to tubes containing 10 ml. of Oeding medium,[7] which is composed of 1 part of bank blood and 3 parts of water. The remaining three slides were each transferred to slants of Tarshis blood medium.[8] The slides were transferred in such a manner that the surface containing the dried sediment was in direct contact with the surface of the slant of blood medium, against which it was pressed firmly.

Following incubation in an upright position at 37° C. for seven days, the slides were removed from the liquid medium and surface of the slants and placed along the inner wall of a funnel which had been inserted in a 2-liter Erlenmeyer flask containing 5 per cent phenol. A cover in which water could enter from the top was then placed on the funnel, and the slides were washed gently by a stream of distilled water. After washing, the slides were transferred to Petri plates with a designated number and autoclaved at 15 pounds for fifteen minutes. The slides were then marked and stained by the Ziehl-Neelsen technique. The low-power objective was used for microscopic examination of the slides. Following removal of the slides from the slants of Tarshis blood medium, the latter were reincubated for development of gross colonies.

Stained smears of the sediment from all specimens which were being examined by the microculture method were examined microscopically. Duplicate cultures were prepared on both Tarshis and Löwenstein-Jensen media, in which 0.2 ml. of the sediment was used for the inoculation of each tube of medium. The cultures were incubated at 37° C. for a period of eight weeks.

A total of 235 sputum specimens was examined in this study. All specimens were collected from patients prior to institution of therapy.

When microorganisms were present in the specimen in sufficient number to be observed in direct smears, microcolonies of *M. tuberculosis* could usually be detected within three days. The microcolonies were well developed after seven days and could be distinguished with the low-power objective of the microscope. However, the number of microcolonies on the slide and, in turn, the ease with which they could be found microscopically were dependent upon the initial number of microorganisms originally present in the specimen. Microcolonies of *M. tuberculosis* which developed in slide cultures on Tarshis blood agar slants were compact, while the serpentine pattern of growth appeared to predominate on slides cultured in Oeding medium. Slide cultures of H37Rv on Tarshis slants produced compact colonies (figure 1*A*). When 1 ml. of distilled water was added to each tube of Tarshis blood agar prior to slide culture of H37Rv, the microcolonies were predominately serpentine (figure 1*B*).

Results pertaining to the detection of *M. tuberculosis* in a group of 235 clinical specimens by direct smear, slide culture, and routine culture on Tarshis and Löwenstein media are summarized in table 1. It will be observed that an equal number of cultures were obtained on Tarshis and Löwenstein media. *M. tuberculosis* was detected in 32.8 per cent of the specimens by direct smear, 41.3 per cent by slide culture, and 44.3 per cent by routine culture on Tarshis and Löwenstein media. Following removal of the slides and reincubation of the slants for forty-nine days, cultures of *M. tuberculosis* were obtained on all tubes of Tarshis medium on which positive slide cultures had been obtained.

The growth of strains of *M. tuberculosis* on primary isolation by slide culture and on Tarshis and Löwenstein media is recorded in table 2. Four strains of tubercle bacilli grew on slide culture but failed to grow on routine culture, while eight strains grew on routine culture on both Tarshis and Löwenstein media but failed to grow on slide culture.

In a comparison of the isolation of *M. tuberculosis* by slide culture in Tarshis and

[7] Oeding, P.: Acta tuberc. Scandinav., 1950, *24–25*, 208.

[8] Tarshis, M. S., *et al.*: J. Bact., 1953, *66*, 448.

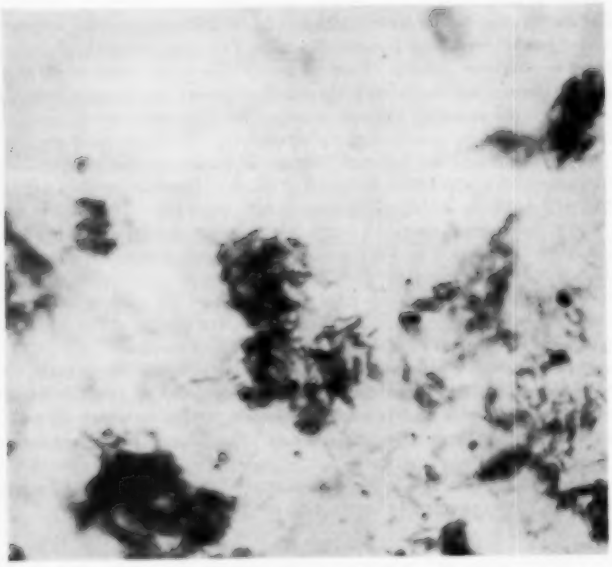

FIG. 1A. Typical compact microcolonies of *M. tuberculosis* (H37Rv) in slide culture on unaltered Tarshis blood agar slant.

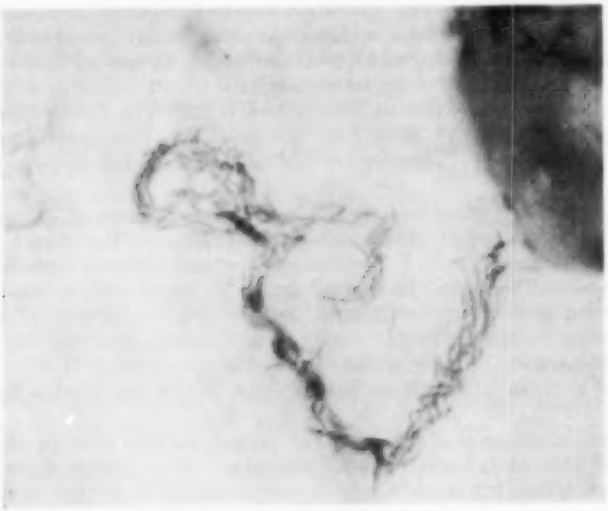

FIG. 1B. Typical serpentine microcolonies of *M. tuberculosis* (H37Rv) in slide culture on Tarshis blood agar slant to which 1 ml. of distilled water had been added prior to culture.

TABLE 1

DETECTION OF M. TUBERCULOSIS BY DIRECT SMEAR, SLIDE CULTURE, AND ROUTINE CULTURE ON TARSHIS AND LÖWENSTEIN MEDIA

Method of Detection	Specimens		
	Total	Positive	
		Number	Per Cent
Over-all testing of specimens	235	112	47.7
Direct smear	235	77	32.8
Slide culture—7 days	235	97	41.3
Slant reincubated for 49 days	234	96	41.0
Tarshis medium—56 days	235	104	44.3
Löwenstein medium—56 days	235	104	44.3

TABLE 2

GROWTH OF M. TUBERCULOSIS ON PRIMARY ISOLATION BY SLIDE CULTURE AND ROUTINE CULTURE ON TARSHIS AND LÖWENSTEIN MEDIA

Group	Positive Specimens (112)	Slide Culture (7 Days)	Tarshis Medium (56 Days)	Löwenstein Medium (56 Days)
A	93	+	+	+
B	4	+	−	−
C	8	−	+	+
D	3	−	+	−
E	3	−	−	+

TABLE 3

ISOLATION OF M. TUBERCULOSIS BY SLIDE CULTURE IN TARSHIS AND OEDING MEDIA

Culture Medium (Slide Culture)	Specimens		
	Total	Positive	
		Number	Per Cent
Tarshis medium	235	95	40.4
Oeding medium	235	85	36.2
Positive Tarshis, negative Oeding	235	12	5.1
Negative Tarshis, positive Oeding	235	2	0.9

Oeding media, 4.2 per cent more cultures were obtained in the former medium (table 3). All cultures of *M. tuberculosis* which were isolated on Tarshis and Löwenstein media were typical and gave positive reactions in the neutral red test.[9]

In the development of this method of slide culture, there was considerable deviation in the regularity with which *M. tuberculosis* could be cultured. The irregularities were found to result mainly from the death of microorganisms during the twenty-hour period required for drying the alkali-digested specimens on the slides. Reproducible results were enhanced when variations were minimized by: careful adjustment of the hydrogen ion concentration of the digested and concentrated specimens; "washing" with 1 per

[9] DUBOS, R. J., AND MIDDLEBROOK, G.: Am. Rev. Tubere., 1948, *58*, 698.

cent Trypticase® broth to reduce the amount of accumulated inorganic salts prior to drying of the specimen on the slide and also to provide a suitable suspending medium for the organisms; and the use of absorbent filter paper to reduce the relatively long period required for drying the specimens on the slide.

The use of a solid rather than a liquid medium in slide culture retains the chief advantage of rapid cultural detection of *M. tuberculosis* and, in addition, allows recovery of the isolated organisms. The blood agar slants may be reincubated after removal of the slide and subsequent observations and studies may be made with respect to characterization of gross colonies.

In summary: A simplified slide culture method for primary isolation of *Mycobacterium tuberculosis* has been described in which Tarshis blood agar was utilized. Microcolonies of this organism were well-developed at seven days, when the results of the slide culture were recorded. *M. tuberculosis* was detected in 41.3 per cent of the specimens by slide culture and 44.3 per cent by routine culture on Tarshis and Löwenstein media. Cultures of *M. tuberculosis* were obtained on all reincubated slants of Tarshis medium which had supported positive slide cultures. Tarshis blood agar slants were found to be superior to Oeding liquid medium for slide culture of *M. tuberculosis*. This study was made on material in which acid-fast bacilli were observed on direct smear in 32.8 per cent of the specimens.

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THE EFFECT OF TUBERCULIN UPON THE OXYGEN UTILIZATION OF BLOOD AND OF SPLENIC TISSUE FROM TUBERCULOUS AND NORMAL GUINEA PIGS[1]

Various tuberculins may exert a specific toxic effect upon tissues extirpated from tuberculin-sensitized hosts. This cytotoxicity is classically demonstrated by the use of tissue culture techniques.[2-4] More recently, leukocytolysis has been observed when peripheral blood obtained from some "tuberculin-sensitive" donors[5, 6] was incubated with tuberculin *in vitro*.

Other techniques for the detection of leukocyte damage have been applied in an attempt to avoid some of the inaccuracies inherent in leukocyte counting. Both the lysozyme release studies of Kerby and Chaudhuri and the metabolic studies of Marks and James failed to demonstrate that tuberculin was toxic for leukocytes from sensitized donors.[7, 8]

This study was undertaken to evaluate the use of oxygen consumption as a measure of the cytotoxicity of tuberculin in small quantities for the leukocyte from the "tuberculin-sensitive" host.

Subjects: Mature guinea pigs were injected intraperitoneally with 0.3 to 1.0 ml. of standard BCG vaccine.[9]

Tuberculin: Skin tests were carried out, using 0.1 ml. of second-strength PPD (equivalent to 0.005 mg. of standard tuberculin).[10] These were graded at forty-eight hours on the basis of the mean diameter of induration as follows: <5 mm. = 0; 5 to 10 mm. = ±; 10 to 15 mm. = +; >15 mm. = 2+; >15 mm. with necrosis = 4+. A pure PPD (Lot No. 02159-F) was used in most of the *in vitro* studies. In a few studies the standard skin-testing tablets which contained "filler" were used.

Tissues: Heparinized blood was obtained by cardiac puncture at intervals from 3.5 to 77 days after inoculation with BCG. The spleens were removed aseptically immediately after the animals had been sacrificed. These were placed directly into iced Tyrode solution and teased apart. To the suspension of cells, freed of tissue stroma, were added: streptomycin, 100 γ per ml.; penicillin, 10 units per ml.; and heparin, 1 mg. per ml.

Metabolic studies: Oxygen consumption by whole blood or splenic cell suspensions was measured. Three milliliter aliquots of blood obtained from a single donor were placed into each of two sterile Warburg flasks. One-half milliliter of Tyrode solution was then added to the cell suspension. The Tyrode solution was modified to contain 0.3 gm. of glucose per 100 ml. and buffered with $NaHCO_3$ to avoid phosphate ions which might alter tuberculin cytolysis.[5] The side arm of the test flask contained 0.1 ml. of Tyrode solution with 50 to 250 γ of PPD; the side arm of the control flask contained 0.1 ml. of plain Tyrode solution. Most studies were carried out using 50 γ of PPD which was approximately the amount used

[1] Supported by grants H-1074(C3) and E-32(C5) from the United States Public Health Service.

[2] Rich, A. R., and Lewis, M. R.: Bull. Johns Hopkins Hosp., 1932, *50*, 115.

[3] Aronson, J. D.: J. Exper. Med., 1931, *54*, 387.

[4] Heilman, D. H., *et al.*: Am. Rev. Tuberc., 1944, *50*, 344.

[5] Favour, C. B.: Proc. Soc. Exper. Biol. & Med., 1947, *65*, 269.

[6] Waksman, B. H., and Bocking, D.: Am. Rev. Tuberc., 1954, *69*, 1002.

[7] Kerby, G. P., and Chaudhuri, S. N.: J. Lab. & Clin. Med., 1953, *41*, 632.

[8] Marks, J., and James, D. H.: J. Hyg., 1953, *51*, 340.

[9] BCG was supplied through the courtesy of Dr. J. D. Aronson, Henry Phipps Institute, Philadelphia, Pennsylvania.

[10] Tuberculin PPD was obtained through the courtesy of Parke, Davis and Company, Detroit, Michigan.

in previous cytolysis studies.[6] Manometer readings were recorded at thirty-minute intervals. After measuring oxygen consumption over a two-hour control period, the side arms were emptied into either the blood or splenic cell suspension, and oxygen consumption was studied over a subsequent two-hour period.

Cytolysis tests: Heparinized blood was collected from single donors, and a mixture of streptokinase (100 units)–streptodornase (10 units) was added to diminish clumping of the leukocytes. Aliquots of 3.5 ml. were pipetted into each of two siliconed test tubes (12.5 by 100 mm.). To one were added 100 γ of PPD dissolved in 0.1 ml. of Tyrode solution; to the other, 0.1 ml. of plain Tyrode solution. The tubes were mixed and leukocyte counts were immediately withdrawn. These tubes were then placed in an incubator at 37°C. on a mechanical roller and turned at 25 r.p.m. After ninety minutes, leukocyte counts on each tube were repeated.

Anisotonia: To obtain standardized percentages of lysed cells, anisotonia was produced. Whole blood was diluted: (*1*) with an equal volume of either distilled water (pH 6.7) or isotonic saline (pH 6.95); or (*2*) with two volumes of water or saline to one volume of blood. Aliquots (3.5 ml.) were then studied for oxygen consumption over a four-hour period.

The osmotic pressure of plasma diluted in the same way as whole blood was determined by freezing point depression.

Oxygen consumption ratio: Oxygen consumption often varied between duplicate flasks. To correct for these nonspecific variations, an "oxygen consumption ratio" between test and control flasks was calculated as follows:

$$\frac{O_2 \text{ consumption-control-before PPD}}{O_2 \text{ consumption-test-before PPD}} \times \frac{O_2 \text{ consumption-test-after PPD}}{O_2 \text{ consumption-control-after PPD}}$$

Values of 1.0 indicated no change; greater than 1.0, an increase in oxygen consumption after the addition of PPD; and less than 1.0, a decrease in oxygen consumption after PPD had been added.

The uptake of oxygen by whole blood and splenic cell suspensions from both normal and "tuberculin-sensitive" guinea pigs was not altered by the addition of tuberculin (table 1).

Simultaneous determination of leukocytolysis and oxygen consumption in a single test vessel was not possible because of the tendency of leukocytes to clump when shaken in Warburg vessels. However, leukocytolysis studies were done on 7 animals with one aliquot of blood, while at the same time oxygen utilization studies were performed with a second aliquot (table 2). Leukocytolysis tests were performed on other BCG-infected guinea pigs as well as on normal control animals.

TABLE 1

EFFECT OF TUBERCULIN UPON OXYGEN CONSUMPTION

Experimental Condition	Tissue Studied	Animals	Experiments	Skin Sensitivity	Oxygen Consumption Ratio
		(*number*)	(*number*)		(*mean* ± 1 S.D.*)
Normal	Blood	24	24	0	0.958 ± 0.294
BCG-infected	Blood	12	49	0 to 4+	1.039 ± 0.258
			11	0	1.041
			12	±	1.052
			11	+	1.013
			4	2+	1.068
			11	4+	1.043
BCG-infected	Spleen	9	20	2+ to 4+	1.049 ± 0.170

* S.D.—standard deviations

TABLE 2

EFFECT OF TUBERCULIN UPON LEUKOCYTE COUNTS

Experimental Condition	Animal Number	Skin Sensitivity	Leukocyte Count Lysis (Per Cent Change)
Normal	13*	0	−0.3 ± 8.46†
BCG-infected	466§	4+	−1.7
	473§	4+	−21.3‡
	475§	4+	−5.6
	469§	4+	−2.1
	TN§	4+	+2.1
	BN	4+	−6.3
	WE§	4+	−12.7‡
	482	4+	−30.1‡
	484§	4+	−30.7‡
	485	±	−16.8‡
	BR	±	−4.8
	479	±	−1.3

* Total number of animals

† Mean ± 2 standard deviations

‡ Values differing more than 2 standard deviations from mean of the normal animals

§ Simultaneous cytolysis and oxygen uptake studies

TABLE 3

EFFECT OF VARIATIONS IN TONICITY UPON OXYGEN CONSUMPTION AND LEUKOCYTE COUNTS

Factor Studied	Number of Experiments	Change After Four Hours Incubation (Mean Percentage)			
		Water (Dilution)		Saline (Dilution)	
		1:1	2:1	1:1	2:1
Leukocyte count	9	−37.8	−63.7	+0.4	−1.3
Oxygen consumption*	9	−57.2	−76.8	+7.2	+38.2

* Comparison of change in oxygen consumption between the second one-half-hour period and the eighth one-half-hour period

Significant decreases in leukocyte counts following exposure to tuberculin were noted in 5 of the 12 "tuberculin-sensitive" animals. Thus, cytolysis as determined by cell enumeration can be demonstrated in BCG-infected guinea pigs. Oxygen uptake studies were repeated thirteen times on blood from 3 of the animals with "positive" leukocytolysis tests. The oxygen consumption ratios of whole blood from these animals did not vary as much as one standard deviation from the mean established in control animals. No correlation existed between leukocytolysis and change in oxygen consumption when both were determined simultaneously.

The validity of alteration in oxygen utilization as a measure of the number and integrity of leukocytes was evaluated by studying the effects of anisotonia on the leukocyte counts and oxygen utilization of whole blood. Leukocytes can exist in a range of tonicity from 220 to 380 milliosmoles (mOs.).[11] The osmolar pressure in a representative study was as follows: Water-plasma (W.-P.) 1:1—135.1 mOs., W.-P. 2:1—89.9 mOs., saline-plasma (S.-P.) 1:1—281.3 mOs., and S.-P. 2:1—285.3 mOs. These extremes of hypo-

[11] TULLIS, J. L.: Am. J. Physiol., 1947, *148*, 708.

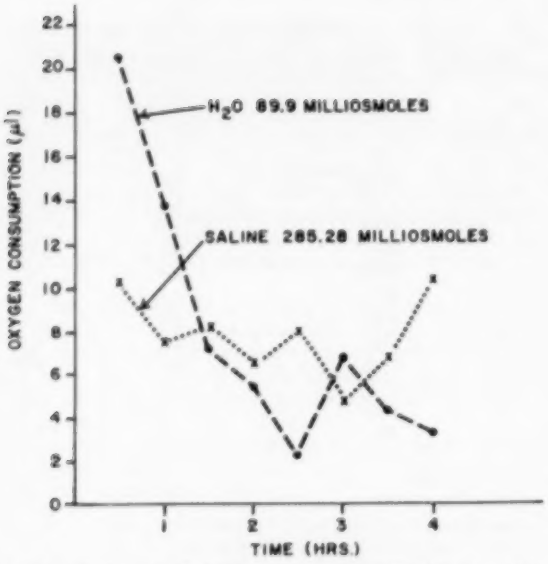

FIG. 1. Effect of variations in tonicity upon oxygen utilization by whole blood (guinea pig).

tonicity caused hemolysis and marked leukocytolysis which were associated with changes in the rate of oxygen utilization (table 3, figure 1). "Oxygen consumption ratios" between hypotonic and isotonic preparations were calculated. The mean values were: 1:1 dilution—0.625; and 2:1 dilution—0.376. These compared with decreases in leukocyte counts of 37.8 per cent and 63.7 per cent, respectively.

* * * *

The exposure of whole blood obtained from "tuberculin-sensitive" guinea pigs to small quantities of tuberculin *in vitro* resulted in significant decreases in the leukocyte counts in 5 of 12 animals. Exposure of whole blood or splenic cell suspensions from these as well as from additional "tuberculin-sensitive" guinea pigs to tuberculin resulted in no significant variations in oxygen utilization.

The lysis of whole blood by extreme hypotonicity resulted in variations in the rate of oxygen uptake which roughly correlated with the fall in leukocyte count. Likewise, oxygen consumption ratios were significantly decreased with the degree of hypotonicity produced.

Based upon rates of oxygen utilization by leukocyte suspensions, Mark and James concluded that tuberculin was not directly toxic to sensitized leukocytes.[5] The present studies indicated that, while changes in oxygen utilization did occur with extreme hypotonicity, similar changes in oxygen utilization did not occur following the addition of tuberculin to whole blood samples which demonstrated leukocytolysis as determined by cell enumeration. Why leukocytolysis, as determined by cell enumeration, was not associated with changes in oxygen utilization was not evident in these studies. However, failure to demonstrate a change in oxygen utilization by sensitized leukocytes following exposure to tuberculin does not seem to warrant a conclusion that tuberculin is not directly toxic to sensitized leukocytes.

In summary: By the techniques used, tuberculin did not alter significantly oxygen uptake by whole blood or splenic cell suspensions obtained from normal or BCG-infected guinea pigs. Significant leukocytolysis was seen following the exposure of whole blood obtained from BCG-infected guinea pigs to tuberculin when studied by the method of cell enumeration. Extreme hypotonicity resulted in variation in rate of oxygen uptake which correlated roughly with the degree of leukocytolysis produced.

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BRONCHOSCOPY IN THE DIAGNOSIS AND THE LOCALIZATION OF BACTERIOLOGICALLY POSITIVE TUBERCULOUS LESIONS

Several workers have reported that secretions aspirated bronchoscopically are more likely to contain tubercle bacilli than expectorated specimens and, further, that it is possible by this method to determine accurately the side of origin of tubercle bacilli.[1-5] Some writers, however, dissent from this position. Tison,[6] for example, found that bronchoscopy is not accurate in localizing the side of origin of tubercle bacilli because the sputum represents a mixture derived from both lungs. It is believed that the conflicting results might be due to inadequately controlled studies. It is the purpose of this report to present the bacteriologic findings in 44 bronchoscopies as compared with the results of examination of expectorated sputum collected over a period of seventy-two hours previous to bronchoscopy. In addition, the accuracy of bronchoscopy in localizing the source of the tubercle bacilli in the sputum was checked in 20 instances in which the source could be determined by other means. In 2 patients there was roentgenographic evidence of appreciable disease on one side only and, in 18, subsequent surgical excision followed by a disappearance of tubercle bacilli from the sputum was confirmatory.

The patients were premedicated with suitable doses of sodium pentobarbital, meperidine hydrochloride, and atropine. When sputum was copious, postural drainage was encouraged prior to the procedure. After topical tetracaine hydrochloride anesthesia to the hypopharynx and tracheobronchial tree, the bronchoscope was passed. Secretions were aspirated from the trachea above the main coryna and the suction tip was discarded. The bronchoscope was then advanced into the side thought to harbor no source of tubercle bacilli. Using a new sterile aspirator, secretions were obtained opposite the upper lobe orifice unless predominant disease was present in another lobe, in which case secretions from the latter were collected. The aspirating cannula was then rinsed with a few drops of physiologic salt solution. If no sputum was noted in the collecting (Lukens) tube, approximately 2 or 3 ml. of physiologic salt solution were injected through the bronchoscope and reaspirated after the patient had been encouraged to cough. Following this the bronchoscope was inserted into the other side and sputum was collected opposite the orifice of the suspected lobe, using a new sterile aspirator. Lavage was performed, if necessary, as on the opposite side. Although curved aspirating cannulas were used in a number of cases, they did not prove to be of greater value than the straight ones and were therefore abandoned.

The specimens were labeled to indicate the side of origin. Films of the concentrated sediment were stained by the Ziehl-Neelsen technique and the number of bacilli was reported by Gaffky count. Cultures were read after six weeks and were reported as 1- to 4-plus or by colony count.

Eleven bronchoscopies were performed on patients whose expectorated sputum was negative for tubercle bacilli. Only one of these yielded bacilli on examination of the bronchoscopic specimens. Bronchoscopic aspirates obtained from the remaining 33 patients with positive expectorations yielded tubercle bacilli in only 28 instances. This indicates that the seventy-two-hour expectoration is more likely to yield tubercle bacilli than the material aspirated bronchoscopically.

[1] Triboulet, F., *et al.*: Rev. de la tuberc., 1931, *12*, 71.
[2] Shipman, S. J.: Am. Rev. Tuberc., 1939, *39*, 629.
[3] Lemoine, J. M., and Diacoumopoulos, A.: Rev. de la tuberc., 1943, *8*, 117.
[4] Bariety, M., and Paillas, J.: Rev. de la tuberc., 1944–1945, *9*, 337.
[5] Warnery, M.: Rev. de la tuberc., 1947, *11*, 695.
[6] Tison, F.: Semaine d. hôp. Paris, 1947, *23*, 2633.

Of the 20 bronchoscopies performed on patients whose side of origin of the tubercle bacilli could be determined by other means, 9 yielded negative smears and cultures on the side opposite the one from which the bacilli originated. In 10 instances, although the opposite side yielded bacteriologically positive results, the side from which the bacilli originated yielded a larger number of organisms by smear and a higher colony count by culture. In one instance the side from which the bacilli originated yielded a lower count by smear and culture.

The following case is presented as an example of the value of bronchoscopy in the localization of the source of tubercle bacilli.

M. F., a 42-year-old white male, developed pulmonary tuberculosis in 1938. Roentgenograms revealed a cavity in the right lung. The patient was treated with left pneumothorax from 1938 until 1943 and did well until March, 1948, when serial roentgenograms revealed an increase in disease, and culture of the gastric lavage was positive for tubercle bacilli. Right pneumothorax was instituted. The patient again did well until July, 1952, when roentgenograms revealed an increase in disease in the left pulmonary apex and the sputum again became positive for tubercle bacilli. In August, 1952, he was started on multiple-drug chemotherapy, but he continued to excrete tubercle bacilli and the strain was resistant to streptomycin, para-aminosalicylic acid (PAS), and isoniazid. On July 23, 1953, pulmonary decortication and right upper lobectomy were performed and were followed on August 19, 1953, by a five-rib postresection thoracoplasty. In spite of these procedures the sputum remained infectious.

Serial roentgenograms of the chest, including planigrams, revealed an indistinct 0.8 cm. radiolucency in the left apex. On the right side, nodular disease without definite cavity could be identified beneath the thoracoplasty.

Bronchoscopy, performed on November 2, 1954, revealed no abnormality, but abundant secretions were seen to come from the left upper lobe bronchus. Specimens taken opposite each upper lobe bronchus were negative for acid-fast bacilli on microscopy. Culture of the specimen from the right side was negative but the culture from the left side yielded one colony of tubercle bacilli. Bronchoscopy was repeated on December 21, 1954. On this occasion a few organisms were detected, both on microscopy and culture of material from the left side and only by culture of the material obtained from the right side.

It was decided to do a left pulmonary resection. Antituberculous drugs were again started. A wedge resection of the left upper lobe on April 28, 1955, yielded a cavity-bearing specimen. Postoperatively the sputum became negative for tubercle bacilli.

In this case differential bronchoscopy yielded evidence which supported the decision to perform a resection on the left side. The bacteriologic studies and the observation of the origin of sputum at bronchoscopy indicated that the bacilli were originating in the left upper lobe.

* * * *

It is evident from the results of this study that specimens of sputum obtained by bronchoscopy are not more likely to contain tubercle bacilli than expectorated sputum collected over a prolonged period. Bronchoscopy may be of value, however, if for some reason expectoration cannot be obtained. This does not mean that bronchoscopy should not be recommended, since the visual findings may prove of value.

From the results obtained in the 20 cases in which the source of tubercle bacilli was checked by other means, it is obvious that sputum tends to spill to the opposite side. This tendency seems greater when sputum is abundant. In spite of this, however, in 19 of the 20 cases studied, the source of the positive sputum was reflected by a higher number of bacilli demonstrable on microscopy or culture. It may thus be concluded that

bronchoscopy tends to indicate the origin of bacilli when they emanate from only one side. In patients who have bacteriologically positive lesions in both lungs, the bacteriologic findings may only indicate which side is producing the most organisms. Even this may be of value in planning surgical therapy. The results were inaccurate in one instance. The possibility of an error in handling the specimens should at least be considered in this instance. The results of bronchoscopy should be carefully correlated with other clinical and laboratory findings.

In summary: Bronchoscopy in the diagnosis and localization of bacteriologically positive lesions of pulmonary tuberculosis has been evaluated. Sputum obtained by bronchoscopy yields fewer organisms than sputum collected over a prolonged period. In unilateral disease the side which is the source of the bacilli in the sputum is indicated in almost all instances by a higher bacillary count on microscopy and culture. With bilateral infectious lesions, bronchoscopy may indicate only the side from which most of the bacilli emanate.

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September 6, 1955

INTERMEDIARY METABOLISM OF GLUCOSE BY MYCOBACTERIUM SMEGMATIS[1]

Although voluminous literature concerned with the metabolism of the mycobacteria has been published, the intermediary metabolism of glucose by these organisms is not well understood. In an excellent review on this subject, Edson[2] proposed that the Embden-Meyerhof scheme might be in operation for the oxidation of glucose. This proposal was based primarily on the fact that methylene blue is reduced by hexosemonophosphates and hexosediphosphates and the observation that triose phosphate dehydrogenase and enolase have been shown to be present in mycobacteria. However, neither hexokinase nor phosphofructokinase have been shown to be present in enzyme extracts. McLaren and Jann[3] also came to the conclusion that *Mycobacterium* 607 utilized the Embden-Meyerhof scheme in the oxidation of glucose and that glucose-grown cells of *Mycobacterium* 607 did not utilize the hexosemonophosphate shunt pathway. They reported that neither ribose nor 6-phosphogluconic acid was oxidized by glucose-grown *M.* 607, but that they were oxidized by gluconate-grown cells. These are not necessarily valid criteria for excluding the monophosphate pathway since they do not take into account permeability factors which could influence the metabolism of the mycobacteria.

The present studies demonstrate that enzyme extracts of *Mycobacterium smegmatis* contain all of the enzymes of the hexosemonophosphate shunt; however, all of the enzymes necessary for the Embden-Meyerhof scheme could not be detected.

Mycobacterium smegmatis was grown on Youman's modification of Proskauer and Beck medium[4] with 1 per cent glucose substituted for glycerol. Cells were grown for two days, filtered, and washed several times in distilled water containing 0.05 per cent Tween® 80. Cell-free extracts were prepared by grinding the washed organisms in 60-mesh glass, and eluting with 0.05 M Tris, pH 7.5. This extract was centrifuged at 0°C. at 25,000 × G for one hour. Except where indicated, the extract was dialyzed against distilled water for ten hours before use. (Diphosphopyridine nucleotide (65 per cent) and triphosphopyridine nucleotide (70 per cent) were obtained from the Sigma Chemical Company.) Hexokinase was determined by the method of Cardini[5] and aldolase by the method of Sibley and Lehninger.[6] Glucose-6-phosphate dehydrogenase and 6-phosphogluconic acid dehydrogenase were determined by measuring the rate of reduction of triphosphopyridine nucleotide at 340 mu in the Beckman DU spectrophotometer. Gluconokinase was determined by reduction of triphosphopyridine nucleotide in the presence of gluconic acid and adenosinetriphosphate. Phosphohexoisomerase was determined by incubating glucose-6-phosphate with the enzyme extract and determining fructose formed by use of the cysteine-carbazole test of Dische and Borenfreund.[7] Phosphofructokinase was tested by the method of Racker[8] and

[1] This study was aided in part by a research grant (G-3591) from the National Institutes of Health, Public Health Service, and in part by a grant from the Committee on Medical Research of the American Trudeau Society, medical section of the National Tuberculosis Association, made possible by a special bequest from the estate of Grace Velie Harris.

[2] Edson, N. L.: Bact. Rev., 1951, *15*, 147.

[3] McLaren, L. C., and Jann, G. J.: Am Rev. Tuberc., 1955, *71*, 260.

[4] Youmans, G. P.: J. Bact., 1946, *51*, 703.

[5] Cardini, C. E.: Enzymologia, 1951, *14*, 362.

[6] Sibley, J. A., and Lehninger, A. L.: J. Biol. Chem., 1949, *177*, 859.

[7] Dische, Z., and Borenfreund, E.: J. Biol. Chem., 1951, *192*, 583.

[8] Racker, E.: J. Biol. Chem., 1947, *167*, 843.

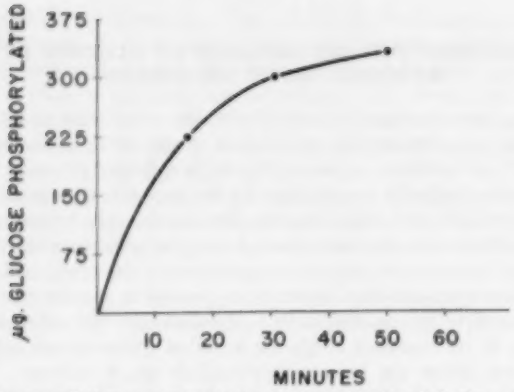

FIG. 1. *Hexokinase:* Tubes contained 0.2 ml. of 0.1 M glycylglycine, pH 7.4; 0.1 ml. of 0.1 M $MgCl_2$; 0.2 ml. of 0.012 M glucose; 0.2 ml. of 0.025 M of adenosinetriphosphate; and 0.4 ml. of enzyme extract (equivalent to 3.2 mg. of protein). At designated times 1.0 ml. of 0.3 N $Ba(OH)_2$ was added, followed by 1.0 ml. of 5 per cent $ZnSO_4$. After centrifugation, residual free glucose was determined on an aliquot of the supernatant according to the method of Nelson.[9] All values were controlled by an identical tube without adenosinetriphosphate.

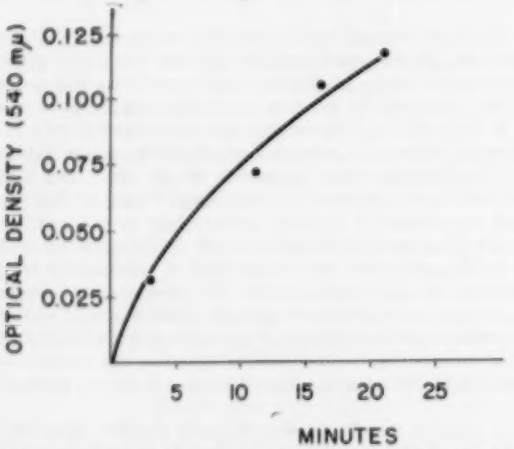

FIG. 2. *Phosphohexoisomerase:* Tubes contained 1.0 ml. of Tris (0.1 M, pH 8.0); 0.2 ml. of 0.05 M glucose-6-phosphate; 0.2 ml. of enzyme extract (0.10 mg. of protein); at indicated times 1.0 ml. of 0.2 N HCl was added to stop reaction, and the resulting ketose was determined on 0.2 ml. aliquot according to the method of Dische and Borenfreund.[7] Glucose-6-phosphate was withheld from control until after addition of HCl.

by the method of Sibley and Lehninger,[8] using fructose-6-PO_4 and adenosinetriphosphate for substrate. Fructose-1-6 diphosphate was used as a control in both cases.

The following enzymes were found in the extracts prepared from glucose-grown *Mycobacterium smegmatis:* hexokinase (figure 1); phosphohexoisomerase (figure 2); glucose-6-,

[9] NELSON, N.: J. Biol. Chem., 1944, *153*, 375.

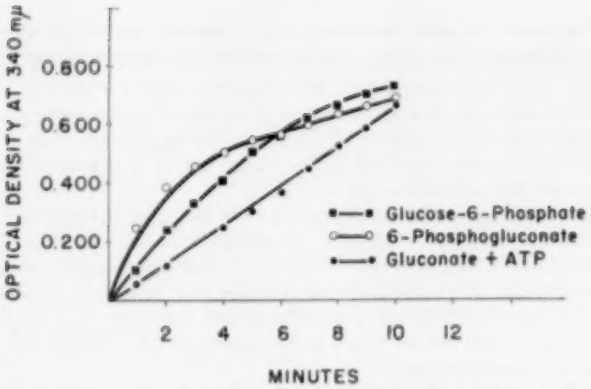

FIG. 3. Silica cuvettes contained 1.0 ml. of 0.1 M Tris, pH 8.0; 0.1 ml. of 0.1 M $MgCl_2$; 0.5 ml. of triphosphopyridine neucleotide (1 mg. per ml.); 0.3 ml. of 0.05 M substrate; and 1.0 ml. of enzyme extract (10 mg. per ml. of protein). Controls without coenzyme and with diphosphopyridine neucleotide in place of triphosphopyridine neucleotide were inactive.

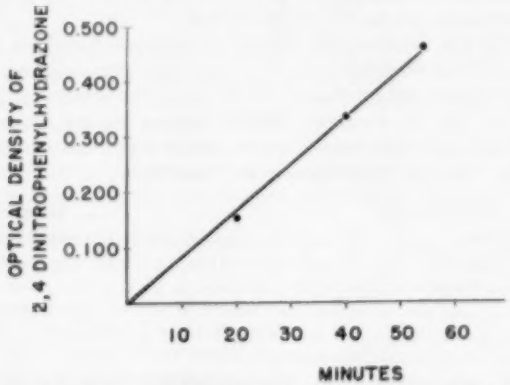

FIG. 4. *Aldolase:* Tubes contained 0.1 ml. of 0.05 M fructose 1, 6 diphosphate; 1.0 ml. of 0.1 M Tris, pH 8.0; and 0.1 ml. of enzyme extract (1.1 mg. of protein). Reaction was stopped at designated times and the 2,4 dinitrophenylhydrazone of triose phosphate was prepared according to the method of Sibley and Lehninger.[6]

phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and gluconokinase (figure 3); and aldolase (figure 4). In addition, ribose-5-phosphate was rapidly cleaved to triose phosphate by extracts of glucose-grown cells. Phosphofructokinase could not be found in these extracts.

The growth of *M. smegmatis* on Proskauer and Beck medium containing glucose as the sole carbon source occurred only after a lag of six to seven days when inoculated with cells grown on glycerol. Once the cells were "adapted" to glucose, rapid growth on Proskauer and Beck medium plus glucose took place. This was unexpected inasmuch as glycerol-grown cells were shown to contain hexokinase, glucose-6-phosphate dehydrogenase, phosphohexoisomerase, 6-phosphogluconic acid dehydrogenase, aldolase, and gluconokinase. Moreover, growth on either fructose or gluconate did not show a de-

monstrable lag under the same conditions, since maximum growth on these substrates was obtained within two to three days after inoculation from glycerol-grown *M. smegmatis*.

* * * *

The results demonstrate that *M. smegmatis* is capable of metabolizing glucose via the hexosemonophosphate shunt pathway. The absence of phosphohexokinase in the extracts suggests that the Embden-Meyerhof pathway may not be in operation but, like most negative evidence, this evidence is not conclusive.

Unpublished observations in which glucose-1-C^{14} was used indicate that only about 10 to 15 per cent of the C^{14} is reclaimed in the metabolic carbon dioxide produced during a resting cell oxidation. This does not exclude the hexosemonophosphate shunt, however, as the sole mechanism of glucose oxidation. Glucose may be directly assimilated, or the end product of decarboxylation of 6-phosphogluconic acid may be a one carbon compound other than carbon dioxide which is reincorporated into cell material as has been proposed for the propionibacteria.[10]

The long adaptive lag of *M. smegmatis* for growth on glucose-containing media remains unexplained. The observation that glycerol-grown cells of *M. smegmatis* contained the necessary enzymes to convert glucose to triose phosphate suggested that adaptation in this instance might involve permeability of the cell membrane. This was further supported by the observation that glycerol-grown cells would grow on fructose or gluconic acid without a demonstrable lag or adaptive period.

In summary: Cell-free extracts of *Mycobacterium smegmatis* have been shown to possess hexokinase, phosphohexoisomerase, glucose-6-phosphate dehydrogenase, 6-phosphogluconic acid dehydrogenase, gluconokinase, and aldolase. The presence of these enzymes is taken as evidence that *M. smegmatis* oxidizes glucose via the hexosemonophosphate shunt. Since phosphofructokinase could not be detected in these extracts, the Embden-Meyerhof pathway may not be operating in this organism.

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October 17, 1955

[10] Wood, H. G., and Leaver, F. W.: Biochim. biophys. Acta, 1953, *13*, 207.

[11] The technical assistance of Miss Lucy Byrd Pegau and Mrs. Margaret Layton is gratefully acknowledged.

THE DECOMPOSITION OF ANTITUBERCULOUS CHEMOTHERAPEUTIC COMPOUNDS WITH REFERENCE TO SUSCEPTIBILITY TESTS

Despite the awareness that certain antituberculous compounds decompose during the inspissation process, the writers believe that the possibility of further degradation of such unstable compounds during incubation at 37.5° C. has not been adequately appreciated. The experience of this laboratory with amithiozone, in which instance the drug underwent an approximately 60 per cent loss during a forty-four-day incubation period subsequent to inspissation, prompted similar investigations with respect to streptomycin, para-aminosalicylic acid (PAS), isoniazid, and cycloserine. The resulting data are presented herein, and it is believed that the implications are of significance in the interpretation of drug-susceptibility tests.

The methods and materials employed were as follows:

Ten microgram solutions of amithiozone[1] per ml. at pH 6.50 were analyzed directly in distilled water by spectrophotometric observation at 321.5 mu. The method of Hendricks and associates[2] was used on homogenized American Trudeau Society (ATS) medium.

One hundred microgram solutions of streptomycin[3] per ml. at pH 6.53 were analyzed in distilled water by the method of Titus and Fried.[4]

One hundred microgram solutions of PAS[5] per ml. at pH 6.53 were spectrophotometrically analyzed in distilled water at 264.5 mu and also with Marshall's modification of Bratton and Marshall's[6] technique for sulfonamides.

Ten microgram solutions of isoniazid[7] per ml. of medium were spectrophotometrically analyzed in distilled water at 263 mu.

Twenty microgram solutions of cycloserine[8] per ml. were analyzed directly in distilled water by spectrophotometric observation at 222 mu and also by a modified Jones[9] method, with the further modification that reading was done at 625 mu instead of at the stated 645 mu.

Optical densities of the solutions were measured in the Beckman spectrophotometer, model DU. Other experimental conditions, such as inspissation, subsequent refrigeration or incubation, duration of experimental observation, and the number of regular interval analyses during the course of this observation, are indicated for the particular solution in the accompanying table. Solution pH values were in all cases adjusted to closely approximate those of the media recommended for drug-susceptibility studies with the compound under investigation.

The measured losses during inspissation as a percentage change from the initial concentration are listed in table 1.

Degradation velocity constants under incubation in all cases were found by graph and application of formula to follow a first order reaction. Thus, for ease in interpretation, *k*

[1] Manufactured as Tibione by Schenley & Company, Lot #848102.

[2] Hendricks, F. D., *et al.*: Tr. Ninth Streptomycin Conference, Veterans Administration, 1950, p. 160.

[3] Streptomycin sulfate, manufactured by Merck & Co., Lot #03B0129.

[4] Titus, E., and Fried, J.: J. Biol. Chem., 1948, *174*, 57.

[5] $NaPAS{\cdot}2H_2O$, manufactured by American Cyanamid Company, Lot #11902-70.

[6] Bratton, A. C., and Marshall, E. K., Jr.: J. Biol. Chem., 1939, *128*, 537.

[7] Manufactured as Rimifon by Hoffman LaRoche, Inc., Lot #8.

[8] Manufactured by Commercial Solvents Company, Lot #R-1.

[9] Veterans Administration Pulmonary Protocol, 1*A*, Pilot Study IX: Cycloserine, February 28, 1955, modified from L. R. Jones, Commercial Solvents Corporation.

TABLE 1

STABILITY OF ANTITUBERCULOUS CHEMOTHERAPEUTICS UNDER INSPISSATION
In Distilled Water* (85°C., 30 minutes)

Compound	Initial Concentration	pH	Number of Analyses†	Method of Analysis	Average Decomposition	Standard Deviation
	γ *per ml.*				*per cent*	*per cent*
Amithiozone*	10	6.75	2	Chemical‡	16.8	±1.60
Streptomycin	100	6.53	2	Chemical	0.0	-
PAS	100	6.53	4	Physical§	1.0	±0.91
PAS	100	6.53	4	Chemical	6.0	±1.80
Isoniazid	10	6.58	4	Chemical	4.8	±0.61
Cycloserine	20	7.03	2	Physical	4.6	±0.18
Cycloserine	20	7.03	2	Chemical	7.8	±1.10

* In the amithiozone experiment the compound was incorporated in American Trudeau Society medium.

† Each analysis was performed in triplicate.

‡ Chemical denotes spectrophotometric analysis with prior chemical procedures, as indicated in the body of the paper.

§ Physical denotes direct spectrophotometric analysis in the ultraviolet without prior chemical procedures.

values (degradation per unit time) have been calculated in table 2 as percentage loss of compound per day. The number of days required for loss of one-half of the original concentration is listed in the column under "Half-Life."

The stability under refrigeration conditions is of interest in reference to either analytical standards or media which have been placed in storage for future use.

The extent of loss for any incubation period can be calculated by substitution in the formula $k = \frac{2.303}{t} \log \frac{Co}{C}$, where k is the velocity constant as recorded in table 2, t equals time in days, Co represents the original concentration of the compound being used, and C the concentration which remains after the required incubation time.

Other published data indicate that degradation rates may decrease with increase in original concentration of the drug. Cummings and associates[10] observed this reciprocal relationship with cycloserine.

Among the factors complicating the interpretation of drug-susceptibility tests with *Mycobacterium tuberculosis* is the proportion resistant to susceptible bacilli. This includes such problems as whether the drug-susceptibility test inoculum comes directly from the sputum specimen or from a culture from this specimen; whether the size of the inoculum has been standardized; and whether the sputum sample is an accurate reflection of the distribution ratio of resistant to susceptible bacilli throughout the host. In addition, and more pertinent to the present study, the concentration of the drug and the time allowed for growth will affect the number of resistant organisms that manifest themselves on culture. The present study has shown that the concentration of the antituberculous compound incorporated in media used for drug-susceptibility tests can become significantly reduced during the incubation period. Such was the experience with amithiozone, streptomycin, and cycloserine.

[10] CUMMINGS, M. M., *et al.*: Antibiot. & Chemother., 1955, *5*, 198.

TABLE 2

STABILITY OF ANTITUBERCULOUS CHEMOTHERAPEUTICS AT 4°C. AND 37.5°C. In Distilled Water*

Compound	Initial Concentration	pH	Pre-Experimental Treatment	Experiment Temperature	Duration of Experiment	Number of Interval Analyses†	Method of Analysis	Average Rate of Decomposition	Standard Deviation	Half-Life‡
	γ per ml			(°C.)	(d)			per cent/d	per cent/d	(d)
Amithiozone	10.00	6.75	—	4.0	21	2	Physical§	0.20	±0.01	347.0
Amithiozone	10.00	6.75	—	37.5	21	4	Physical	3.70	±0.31	18.7
Amithiozone*	8.32	6.50	Inspissated	4.0	45	5	Chemical‖	0.00	—	—
Amithiozone*	8.32	6.50	Inspissated	37.5	44	4	Chemical	2.45	±0.80	28.3
Streptomycin	100.00	6.53	—	37.5	46	6	Chemical	3.42	±0.32	20.3
Streptomycin	100.00	6.53	Inspissated	37.5	46	6	Chemical	3.25	±0.61	21.3
PAS	100.00	6.53	—	37.5	46	6	Physical	0.07	±0.01	990.0
PAS	99.00	6.53	Inspissated	37.5	46	6	Physical	0.00	—	—
Isoniazid	10.00	6.58	—	37.5	46	6	Physical	0.36	±0.03	192.5
Isoniazid	9.52	6.58	Inspissated	37.5	46	6	Physical	0.19	±0.04	364.7
Cycloserine	20.00	7.03	—	4.0	50	6	Physical	0.07	±0.01	990.0
Cycloserine	20.00	7.03	—	37.5	52	6	Physical	0.73	±0.03	94.9
Cycloserine	18.40	7.03	Inspissated	4.0	50	7	Chemical	0.00	—	—
Cycloserine	18.40	7.03	Inspissated	37.5	50	7	Chemical	0.54	±0.02	128.3

* In the two indicated amithiozone experiments the compound was incorporated in American Trudeau Society medium.

† Each analysis was performed in triplicate.

‡ Physical denotes direct spectrophotometric analysis in the ultraviolet without prior chemical procedures.

§ Chemical denotes spectrophotometric analysis with prior chemical procedures, as indicated in the body of the paper.

‖ Average rate of decomposition was calculated on basis of the formula, $k = \frac{2.303}{t} \log \frac{Co}{C}$; half-life, on the basis of $t\frac{1}{2} = 0.693/k$.

The present study has been concerned with distilled water solutions of these drugs, with the exception of amithiozone. The next logical step would be to assay the compound in culture media by chemical and microbiologic techniques. Nevertheless, evidence does exist that chemical concentration and antibacterial potency or activity in media can, at least in a general fashion, be predicted from results obtained from distilled water solutions. In the case of amithiozone, the decomposition rate in American Trudeau Society medium paralleled that in water.

While it has been found by Regna and associates[11] and in this laboratory that streptomycin decomposes in water solutions, Henry and associates[12] observed it to decrease in activity (biologic assay) in several bacteriologic culture media as well as in human serum

[11] REGNA, P. P., *et al.*: J. Biol. Chem., 1946, *165*, 631.

[12] HENRY, R. J., *et al.*: J. Pharmacol. & Exper. Therap., 1947, *90*, 42.

and human plasma. Drummond and co-workers[13] also discovered that a chemical loss of streptomycin is accompanied by a similar loss in biologic activity with inspissation in Löwenstein medium. These observations are in disagreement with those of the present study in which no loss of streptomycin could be detected in a water solution subjected to inspissation. Moreover, Pyle[14] observed no appreciable diminution in the potency of streptomycin over a seven-week period, nor did Waksman and Schatz[15] observe any decrease in the activity of crude solutions during fifteen to seventeen days at 37° C. However, Fielding's[16] report of late scanty growths on streptomycin-containing medium from pretreatment strains provides strong indication of drug loss and tends to support the contention that drug degradation can permit the growth of the remaining drug-susceptible microorganisms.

In the case of cycloserine, Cummings and associates[10] observed a progressive drop in concentration of the drug, as determined by chemical assay, in a synthetic liquid medium over a fifteen-day period.

Although isoniazid has been generally considered to be relatively stable, it is susceptible to degradation when exposed to heat or on incubation at 37° C. in the presence of trace amounts of free cations like cupric and manganous ions.[17]

Thus, an investigator who is unaware of the extent of losses in the media may report misleading values for drug-resistance concentrations. In view of the above, it is concluded that drug-susceptibility tests, especially on thermolabile compounds, may contribute some information but cannot be considered as the only basis of decision as to further treatment with the chemotherapeutic in question.

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November 18, 1955.

[13] Drummond, M. C., *et al.*: Dis. of Chest, 1951, *19*, 158.
[14] Pyle, M.: Proc. Staff Meet., Mayo Clin., 1947, *22*, 465.
[15] Waksman, S. A., and Schatz, A.: J. Am. Pharm. A., 1945, *34*, 273.
[16] Fielding, J.: Tubercle, 1951, *32*, 310.
[17] Lewin, E., and Hirsch, J. G.: Am. Rev. Tuberc., 1955, *71*, 732.

Correspondence

IRREGULAR DISCHARGE

To the Editor of The American Review of Tuberculosis and Pulmonary Diseases:

My source of factual information on this subject is the admirable pamphlet produced by the Veterans Administration in October, 1948. In its Appendix "D" are listed 68 hospitals with their annual turnover of patients and their annual irregular discharge rate.

If we group these hospitals by their apparent size (judging from their annual turnovers) we find the following:

(*a*) Twenty-one hospitals with turnover < 80 have average irregular discharge rate of 14 per cent

(*b*) Forty-seven hospitals with turnover of 80–700 have average irregular discharge rate of 36 per cent

(*c*) Nine hospitals with turnover of 80–110 have average irregular discharge rate of 28 per cent.

Both of the two latter percentages exceed 14 per cent with astronomical significance, and demonstrate in addition that the turnover figure of 80 is pretty close to the "critical value." When you once get above 80, further increases don't matter much.

Other things being equal, why should smaller establishments show better records than the larger ones? What have they got which the bigger hospitals lack?

The answer, Sir, surely is—Fewer Fellow Patients.

Now after a really good dinner we can kid ourselves that we love 98 per cent of our neighbors; but even then there remains the obstinate 2 per cent, whom we are dismayed to find we cannot tolerate (Day's Law). They are like the mosquitoes at the outdoor "steak broil." If we are at a smallish "steak broil" and get stung by a mosquito, we can take it as all part of the fun; but put us in at a party ten times as large and let us be stung by ten, nine, or even four mosquitoes, and we reach for our hats and make our excuses to our hostess. If, moreover, there is a polite fiction that mosquitoes don't exist, a psychiatrically-minded hostess will be a bit misled by her analysis of our excuses.

For "mosquitoes" read "psychopaths," i.e., anyone who irritates us, and you can see what may happen in large sanatoria. Psychopaths, you must also bear in mind, have the bulge on mosquitoes in that they can, and do, combine forces, and their power to irritate rises exponentially with their raw numbers. A gang of ten can obviously develop more than ten times the nuisance-value that might lie in the gift of any one individual.

Another minor point: We don't feel the same compunction about slinking away from a large impersonal football crowd as we do from a drawing-room piano recital, where we may constitute as much as ten per cent of the audience and our defection would make us conspicuous.

This isn't psychiatry. It's ordinary common sense, so I doubt if anyone will pay any attention to it.

Yours sincerely,
GEORGE DAY

THE SANATORIUM
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November 8, 1955

CONCERNING THE APICAL LOCALIZATION OF POST-PRIMARY PULMONARY TUBERCULOSIS EXPLAINED BY THE SPECIFIC GRAVITY OF TUBERCULOUS MATERIAL

To the Editor of The American Review of Tuberculosis and Pulmonary Diseases:

While primary pulmonary tuberculosis caused by inhalation of tubercle bacilli shows a tendency to be localized to the lower or caudal two-thirds of the lungs, the conditions are otherwise concerning post-primary pulmonary tuberculosis which in adults of the white race commences, as a rule, in the apical segments of the lungs.[1, 2] The generally accepted opinion is that post-primary pulmonary tuberculosis develops hematogenously.

In his comprehensive monograph in 1951 on the pathogenesis of tuberculosis, Rich writes concerning the apical localization of post-primary pulmonary tuberculosis in man, "It is certainly not possible at present to explain satisfactorily the apical localization of the disease in adults," and further, "The present unsatisfactory state of this interesting and important question is evident."

Smith and his co-workers[3-5] have since dealt with the problem. They mention sixteen previous hypotheses and propound a seventeenth theory, "The Stream Flow Theory," according to which the blood is only mixed incompletely in the right side of the heart and passes through in layers so that blood from the superior vena cava mainly reaches the upper lobes of the lungs while blood from the inferior vena cava is conducted mainly to the caudal segments of the lungs. As it is presumed that infectious material from tuberculous lymphatic nodes is conducted via lymph channels and the thoracic duct to the superior vena cava, this theory may explain the apical localization of post-primary pulmonary tuberculosis in man.

The observation that thrombotic infarcts most frequently occur in the basal lobes of the lungs is explained by Smith and his co-workers by the fact that the thrombi originate mainly from the area of drainage of the inferior vena cava.

The apical localization of post-primary pulmonary tuberculosis in man constitutes, however, only part of a more comprehensive problem, viz., the localization of post-primary tuberculosis in the organs in man and animals. The disease attacks electively the upper part of the kidneys in man, and the problem of the basal localization of tuberculous leptomeningitis is still unsolved.[6]

Rothlin and Undritz[7-9] have observed in man and demonstrated in experimental animals that tuberculosis occurring after the development of allergy becomes localized particularly to the spatially uppermost parts of the organs. In pulmonary tuberculosis in man this becomes the cranial segments; in the rabbit, the dorso-caudal segments; and in the bat, on account of its peculiar resting position, the caudal segments are predominantly attacked.

The authors find a corresponding localization in regard to the kidneys and also in

[1] Malmros, H., and Hedvall, E.: Studien über die Entstehung und Entwicklung der Lungentuberkulose, Johann Ambrosius Barth, Leipzig, 1938.

[2] Rich, A. R.: The Pathogenesis of Tuberculosis, Charles C Thomas, Springfield, Illinois, 1951.

[3] Smith, D. T., *et al.*: Am. Rev. Tuberc., 1954, *70*, 547.

[4] Abernathy, R. S., *et al.*: Am. Rev. Tuberc., 1954, *70*, 557.

[5] Bondurant, S., and Smith, D. T.: Am. Rev. Tuberc. 1954, *70*, 570.

[6] Huebschmann, P.: Pathologische Anatomie der Tuberkulose, Julius Springer, Berlin, 1928.

[7] Rothlin, E., and Undritz, E.: Verhandl. d. Schweiz. Naturforsch. Gesellschaft, 1944, *124*, 169.

[8] Rothlin, E., and Undritz, E.: Zeitschr. f. Allg. Path. u. Bakt., 1952, *15*, 690.

[9] Rothlin, E., and Undritz, E.: Bull. schweiz. Akad. d. med. Wissensch., 1954, *10*, 199.

tuberculous meningitis when it is borne in mind that the individual concerned rapidly becomes confined to bed with neck rigidity whereupon the base of the brain assumes a position spatially higher than the convexities.

The experiments by Medlar and Sasano on rabbits[10] and Medlar's investigations on pulmonary tuberculosis in cattle[11] are also in agreement with the recorded rule of localization.

Although it has long been known that the tubercle bacillus, tuberculous pus, and particularly the peripheral zone of caseous lymph node tubercles are very rich in lipoids,[12, 13] no previous theory has connected this with the localization of post-primary tuberculosis.

By adding drops of tuberculous pus from a pleural empyema and a lumbar abscess[14] to solutions of copper sulphate and to mixtures of benzol and chloroform of known specific gravity, I was able to demonstrate that the specific gravity of the tuberculous pus was between 1.010 and 1.015, i.e., less than the specific gravity of serum which lies between 1.025 and 1.030. In agreement with this observation, the tuberculous pus rose to the surface when added to a vessel containing serum.

Staphylococcal pus from a carbuncle showed a specific gravity which was greater than 1.030 and the specific gravity of various fragments of a thrombus[15] from a case of embolism in the pulmonary artery was more than 1.035. In accordance with these observations the materials mentioned sank in serum.

On account of its buoyancy, tuberculous pus in the larger vessels must be supposed to remain in the spatially highest part of the wall of the vessel in which it is conducted by the blood stream.

The state of affairs may be roughly compared with small wax balls passing through glass tubes through which water is conducted. It was easy for me to observe that the particles congregated at the spatially uppermost part of the wall of the tube. The smaller the wax balls were, the more slowly did they move through the tube, an observation which is in agreement with the fact that the rate of flow is greatest in the central part of the tube. When the tube branches, as do the pulmonary arteries, the particles of wax pass through the uppermost branch.

Particles with a specific gravity greater than water congregate at the lower part of the wall of the tube and pass through the lowest branch of the tube. Slight irregularities in the glass tube stop the progress of the smallest particles.

The size also plays a part in the speed with which a particle rises or sinks in a fluid.

If the circulatory system be again considered, it appears probable that the trabeculae and pouches in the inner wall of the heart may obstruct the transport by the blood of particles congregated along the walls. Light particles will, when forced through the pulmonary artery and its branches, on account of their buoyancy, continually force themselves upwards in the arterial system of the lungs and come to rest in the spatially uppermost part—in man, the apices; in rabbits and cattle, the dorso-caudal part of the lungs; and in the bat, the caudal pulmonary lobes.

[10] Medlar, E. M., and Sasano, K. T.: Am. Rev. Tuberc., 1936, *34*, 456.

[11] Medlar, E. M.: Am. Rev. Tuberc., 1940, *41*, 283.

[12] Caldwell, G. T.: J. Infect. Dis., 1919, *24*, 81.

[13] Wells, H. G., *et al.*: The Chemistry of Tuberculosis, Williams and Wilkins Company, Baltimore, 1923.

[14] The writer expresses his gratitude to Dr. Hans Thomsen, Physician-in-Charge, The National Association's Hospital for Surgical Tuberculosis in Juelsminde, for specimens supplied.

[15] The writer is indebted to Dr. Tage Lund, Pathologist, The Pathological Institute, The Municipal Hospital, Aarhus, who kindly supplied the thrombus mentioned.

Corresponding conditions may also play a role in the kidneys and other organs when caseous material containing bacilli is discharged into the blood stream.

The number, size, and bacillary content of the particles also play a part in the distribution and development of the lesions in the organs. Other things being equal, the tissue changes are proportional with the number of bacilli supplied.

The theory outlined above, based upon the specific gravity of embolic material spread by vascular channels, explains the localization of post-primary tuberculosis and is in good agreement with the results of the experiments performed by Smith and his co-workers by intravenous injection of *Lycopodium* and tuberculous pus into rabbits in various positions.

The spores of *Lycopodium* have a high lipoid content which implies that they tend to float upwards in the pulmonary vascular system on account of their buoyancy. The spatial relations of the great pulmonary vessels to one another during the various positions of the rabbit and the period during which the animal is held in an abnormal position in connection with the injections are also instrumental in determining in which lobes of the lungs the infarcts occur. In this connection it must be mentioned that the rabbit will naturally attempt to resume the normal position as rapidly as possible.

When particles of red lead are concerned, they are conducted by the blood stream to the spatially lowest part of the lungs, a fact which Rothlin and Undritz were able to demonstrate by X-ray records *inter alia*. It appears reasonable to assume that the high specific gravity is responsible for this phenomenon.

Similar conditions probably play a part when thrombotic particles are conducted by the blood stream.

The problem concerning the localization of pulmonary tuberculosis of hematogenic origin in infants and Negroes may also be simply solved. In this connection, Rich writes when comparing with white adults, "There is no more puzzling circumstance in the pathogenesis of pulmonary tuberculosis, nor one that has excited more interest and speculation, than the peculiar fact that in the adult the disease begins almost invariably in the upper portion of the upper lobe, whereas in the child it tends to occur indiscriminately in any part of the lung."

Infants constantly adopt a recumbent position and move in the horizontal plane, whereupon pulmonary processes of hematogenous origin, in accordance with the rule of localization and the new theory, do not show any particular localization to the apices but occur equally frequently in all parts of the lungs.

The observation that pulmonary tuberculosis in the Negro frequently assumes a form very reminiscent of the conditions in the infant may be explained by the fact that, according to Rich, the disease in the Negro assumes, as a rule, a strikingly acute character. It is consequently natural that the Negro becomes bedridden earlier than do individuals of the white race and thereafter the situation is identical with that in the infant.

Yours sincerely,

THE NATIONAL ASSOCIATION FOR THE CAMPAIGN AGAINST TUBERCULOSIS
SILKEBORG SANATORIUM, DENMARK
CURT E. TARNOWSKI

December 1, 1955

MODIFIED MICROCOLONIAL TEST FOR VIRULENT MYCOBACTERIA

To the Editor of The American Review of Tuberculosis and Pulmonary Diseases:

In previous publications (Wayne, L. G.: J. Bact., 1955, *69*, 92; Krasnow, I., *et al.*: Am. Rev. Tuberc., 1955, *71*, 361), we described a microcolonial test for virulent mycobacteria

employing a neutral red differential staining technique for colonies grown on molecular filter membranes or filter paper strips. Since that time we have gained more experience with this technique and wish to recommend the use of a modified test based on the best features of the molecular filter and of the filter paper methods.

Molecular filter membranes are cut into 5 mm. by 10 mm. strips, autoclaved at 10 pounds pressure for ten minutes, and placed on the surface of tubed Löwenstein-Jensen medium, in the manner formerly employed with filter paper. Sterilization at higher temperatures or for longer times makes the strips crumble easily. The strips are inoculated by loop or Pasteur pipette and incubated for ten days. The tubes should be capped tightly to prevent drying.

The strips are then removed with sterile forceps and stained in the manner previously described for the molecular filter method.

This modification has the advantage of the simplicity formerly obtained with the paper strip method. Two disadvantages of the paper technique are eliminated by the substitution of molecular filter strips. First, the molecular filter membrane has a homogeneous texture resulting in a uniform background for the examination of microcolonies, whereas the coarse paper fibers are often confusing. Second, there may be a tendency for normally non-corded mycobacteria to grow along paper fibers, resulting in a false impression of cording. The molecular filter material, having no fibers, cannot introduce this source of error.

It has also come to our attention that our directions for the preparation of the NR-6 stain have been subject to misinterpretation, due to an unfortunately ambiguous choice of words in our original descriptions. The statement, "NR-6: a saturated solution of neutral red in 95 per cent ethanol, filtered and acidified with 1 per cent (v/v) of concentrated hydrochloric acid" was intended to convey the idea that 1 ml. of concentrated (12N) hydrochloric acid was added to 99 ml. of the alcoholic dye solution.

Sincerely,

LAWRENCE G. WAYNE

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SAN FERNANDO, CALIFORNIA

December 7, 1955

CHROMOGENIC ACID-FAST BACILLI FROM HUMAN SOURCES

To the Editor of The American Review of Tuberculosis and Pulmonary Diseases:

In correspondence which recently appeared in the REVIEW, Middlebrook and associates[1] raised several questions regarding a report we had published[2] dealing with the *in vitro* susceptibility of a number of chromogenic acid-fast bacilli to several antimicrobial agents. We had demonstrated that under the conditions of the experiment all of the strains were insensitive to isoniazid in concentrations as high as 100 γ per ml. of medium. Middlebrook and his co-workers suggested that the strains we had studied are not pathogenic mycobacteria of the "yellow bacillus" type described by Buhler and Pollack,[3] and Timpe and Runyon,[4] or of those which they had investigated.[1] They pointed out that we had assumed some of our strains to be agents of disease in the patients from whom they were derived, and similar to, or identical with, the "yellow bacilli" described by Buhler and Pollack. They also stated it would seem unwise to conclude that the chromogenic "yellow

[1] MIDDLEBROOK, G., *et al.*: Am. Rev. Tubere., 1955, *72*, 693.
[2] TARSHIS, M. S., *et al.*: Am. Rev. Tubere., 1955, *72*, 119.
[3] BUHLER, V. B., AND POLLAK, A.: Am. J. Clin. Path., 1953, *23*, 363.
[4] TIMPE, A., AND RUNYON, E. H.: J. Lab. & Clin. Med., 1954, *44*, 202.

bacilli" are highly resistant to isoniazid, and that three of their patients infected with such organisms, and treated with isoniazid and pyridoxine, responded both clinically and bacteriologically. Since their review of our work is based on several points of misunderstanding, we think the matter should be clarified.

First, we did not intend to imply that any of the chromogens we had investigated[2, 5] were pathogenic for man, particularly since case histories were either not available or were vague and incomplete. On the other hand, several of the physicians who sent us their strains for study felt strongly that they were probably the cause of disease in their patients, since these were the only acid-fast bacilli which could be isolated and were consistently the only or the predominant bacteria obtained on repeated culture. It was interesting, and suggestive, that these organisms were isolated from patients with, or suspected of having, tuberculosis. It was interesting, too, that some of the organisms caused not only disease but also death in the guinea pig, mouse, and rabbit, although it required much larger inocula of such organisms than was necessary with virulent mycobacteria to produce disease and death in the same animals. When similar amounts of known saprophytic mycobacteria (*M. butyricum*, *M. phlei*, and *M. smegmatis*) were inoculated into these animals under identical conditions, neither disease nor death could be produced. However, this does not preclude the possibility that the same, or other, saprophytic strains can produce disease in animals under different conditions. For example, Schwabacher[6] showed that rabbits may be killed by intravenous inoculation of 10 mg. of either living or heat-killed saprophytic acid-fast bacilli and that guinea pigs given intraperitoneal injections of these organisms mixed with butter or mineral oil may show extensive lesions which closely simulate those of tuberculosis. This observation has been made by others.[7, 8] On the basis of our investigations and after comparison of the strains which we studied with those of others, we concluded that under certain conditions some of the organisms might well be the cause of disease in man.

We would also like to call attention to the fact that differentiation of mycobacteria from the standpoint of pathogenicity is often extremely difficult. This is due to the necessity of qualifying the term "virulence" on the basis of strain characteristics, infective dose, route of inoculation, nutritional state of the animal, species' susceptibility or resistance, the time interval between infection and necropsy, and all other factors which must be considered. While animal testing is one of the most reliable methods available for evaluating the pathogenic potentialities of tubercle bacilli and other mycobacteria, it must not be forgotten that pathogenicity for the animal may be different from that for man. It is, therefore, important to understand the limitations of the animal virulence test as well as the influences which operate to produce variations from the typical characteristics of the mycobacteria.

Second, we re-examined our statement, "Although the problem of the pathogenic potential of atypical mycobacteria is controversial, there is evidence that some of these strains can produce disease in man." We realize the wording is not clear. We were referring here to the chromogens in general and not to the strains investigated by us. However, we recognize now how the reader could conclude that we assumed some of our strains were similar or identical with the "yellow strains" of mycobacteria described by Buhler and Pollack. In fact, all ten strains which we studied were not yellow at all, but intensely

[5] Tarshis, M. S., and Frisch, A. W.: Am. Rev. Tubere., 1952, *65*, 278; 289; 302.

[6] Schwabacher, H.: Medical Research Council: Special Report Series, No. 182, London, His Majesty's Stationery Office, 1933.

[7] Rabinowitsch, L.: Ztschr. f. Hyg. u. Infektionskr., 1897, *26*, 90.

[8] Hagan, W. A., and Levine, P.: J. Am. Vet. M. A., 1932, *81*, 723.

orange. These had been subcultured at least 200 times on various egg media and in Tween®-albumin liquid medium. Several strains were lemon yellow when first isolated, but turned orange on subculture. This could well account for significant alterations in their original characteristics. Although histories of the organisms were given in a previous study,[4] these additional details should have been made clear in our last report. It should also be pointed out that virulent chromogens are not necessarily "yellow" in type. There are a number of reports[5] concerning the pathogenicity of atypical mycobacteria both in animals and in man with strains exhibiting other pigmentation, such as white, pink, orange, and brick-red.

Third, we wish to emphasize that our susceptibility tests were performed under a different set of conditions than those employed by Middlebrook and associates.

Fourth, it is noteworthy that all of the "yellow type" chromogenic bacilli studied by Middlebrook and his associates were susceptible to isoniazid *in vitro* at concentrations between 0.5 and 1.0 γ per ml. It would be valuable to test a greater number of such strains to see if similar results would be obtained. Perhaps the isoniazid susceptibility test may prove to be a helpful tool in distinguishing between some of the pathogenic and nonpathogenic chromogens. But in view of the limitations of the *in vitro* susceptibility test,[6] it is not likely to prove so simple. However, it might be worth while to investigate this aspect. The whole matter of the status of atypical mycobacteria is fraught with many difficulties. Far more work needs to be done to elucidate the many unsolved problems in this field of investigation.

We appreciate the interest shown by Middlebrook and his associates in our report and for calling attention to this matter.

Sincerely yours,
MAURICE S. TARSHIS

DEPARTMENT OF BACTERIOLOGY
UNIVERSITY OF CALIFORNIA
BERKELEY 4, CALIFORNIA

December 7, 1955

VITAMIN A METABOLISM IN TUBERCULOUS PATIENTS

To the Editor of The American Review of Tuberculosis and Pulmonary Diseases:

Since the publication of our paper, "A Physiologic and Clinical Study of Failures in Vitamin A Metabolism in Tuberculous Patients" (Am. Rev. Tuberc., 1955, *72*, 218), we have had several inquiries requesting data for a synthetic vitamin A versus natural vitamin A controversy. We feel this misinterpretation of our data warrants clarification by us.

Neither natural vitamin A nor synthetic vitamin A seems to be the tuberculosis healing factor, as our previous publication in the *Proceedings of the Society for Experimental Biology and Medicine* (1938, *38*, 543) entitled, "Cod Liver Oil Therapy in Experimental Tuberculosis" pointed out. An oil with a high vitamin A content (halibut liver oil), an oil with a high vitamin A and D content (tuna liver oil), and a low potency cod liver oil were used, and only the cod liver oil showed the healing action. Later, a molecular distilled vitamin A (from cod liver oil) and an oil rich in vitamin A_2 (burbot liver oil) were used and both failed to demonstrate the healing action. These last two observations were not published previously but are mentioned now as they may help to clarify the situation.

The tuberculosis healing factor in cod liver oil can be concentrated by saponifying the

[6] TARSHIS, M. S., AND FRISCH, A. W.: Am. J. Clin. Path., 1951, *21*, 729.

oil. The factor is found in the nonsaponifiable residue (NSR) along with vitamins A and D, sterols, hydrocarbons, and other unknown substances. Further chemical and animal studies are in progress, attempting to isolate and identify the active material. Our previous clinical study reported in the REVIEW (1951, *65*, 381) entitled, "A Study of the Relation of Nutrition to the Development of Tuberculosis," as well as the paper under discussion, would indicate that the healing substance is closely associated with vitamin A.

Sincerely yours,

HORACE R. GETZ

THE CHARLES COOK HASTINGS HOME
3800 N. LINCOLN AVENUE
ALTADENA, CALIFORNIA

January 18, 1956

Books

MORBIDITY IN THE MUNICIPAL HOSPITALS OF THE CITY OF NEW YORK. By Marta Fraenkel and Carl L. Erhardt. Pp. 229, Russel Sage Foundation, New York, New York, 1955, Price, $4.50.

This is a statistician's book with tables providing the meat, and the commentary supplying an exposition of method but only a meager interpretation of the data. In this way a wealth of information has been packed into a small compass, a book of only 165 pages without the appendices.

The authors have endeavored to show the value of reporting morbidity data from the municipal hospitals in New York City, and have advanced the thesis that reporting of such information by all hospitals would aid in understanding the prevalence of illness. This would allow for more intelligent planning of facilities for medical care.

In the present study, a cross section of the population cared for in the municipal hospitals during a six-month period is presented. The authors rightly point out that the real value of hospital morbidity reporting lies in a constant reporting system which provides information on which future planning decisions may be soundly based. Nevertheless, the pilot study carried out by Dr. Fraenkel and Mr. Erhardt has tested methods of obtaining data and has proved the feasibility of such reporting. Also, the information developed by the study gives significant insights into the problems of medical care for the medically indigent in New York City. For example, the authors point out that "the fact that 612 aged persons with fracture of the femur had to be cared for in the municipal hospitals within the comparatively short period of six months, indicates that extension of safety measures is an important field for geriatrics."

The chapter on tuberculosis is of particular interest because it has bearing on the changing pattern of hospital care for this disease. Figures show the death rate dropping year by year, with the greatest rapidity since 1950. In addition, the emphasis in institutional care has shifted from out-of-town sanatorium care to hospitalization within the city.

Figures for length of stay of tuberculous patients are of considerable significance for hospital-care planning. The authors found a wide range in lengths of stay, from 15 per cent who stayed less than two weeks to 43 per cent who stayed more than three months. As many as 620 patients stayed more than a year. Patients leaving the hospital against medical advice accounted for most of the short stays.

Deaths were found to account for very few of the discharges among the young and middle-aged patients, but the proportion increased with age. Nearly one-quarter of the patients 65-years-old and more died in the hospital.

Other useful information was obtained for chronic illness in general, and the authors were led to recommend strongly the establishment of a regular morbidity reporting system for all the hospitals in New York City. Since this would be a complex undertaking, they suggest starting with the municipal hospitals and gradually extending it to the others.

Dr. Fraenkel and Mr. Erhardt have made a notable contribution to a science of medical-care planning through their book. The day would almost seem in sight when the needs of the populace for medical care could be accurately anticipated and, because of this advance notice, met with maximum effectiveness.

GEORGE READER
New York, New York

PULMONARY RESECTION FOR TUBERCULOSIS. By Poul Ottosen. Pp. 166, Universitetsforlaget I Aarhus, Aarhus, Denmark, 1955, Price, $3.00.

The monograph is a compilation by the author of his experience as resident on the surgical staff of the Missouri State Sanatorium from 1950 to 1952 and, subsequent to that, his experience in his native Denmark. This work is divided into sections discussing the general survey of patients, bacteriologic status, means of diagnosis, and classification of lesions.

The surgical indications of both the American and Denmark series are set forth. Anesthesia, surgical technique, and, particularly, indications for pneumonectomy, lobectomy, and segmental resection are discussed.

The type and number of complications are discussed and are related to the type of extirpative surgery.

The monograph is 166 pages long, is paper bound, and has a good bibliography.

The roentgenograms, although seemingly well selected, do not reproduce well.

PETER DINEEN
New York, New York

PRACTICAL MEDICAL MYCOLOGY. By Edmund L. Keeney. Pp. 145, Charles C Thomas, Springfield, Illinois, 1955, Price, $3.75.

This lecture series monograph covers the field well in a simple and practical manner. The chapters are arranged according to the etiologic agent, which is a help in so diverse a field. The illustrations are rather diagrammatic, and the nuances of culture reading are lost as a result. The internal medicine aspects of mycology are well covered, especially the pulmonary aspect. Perhaps the author is unduly impressed with the pathogenicity of *Geotrichum* and *Aspergillus* as superficial invaders of the skin, but he should be applauded for rejecting the all too frequent *post hoc* causative reasoning where *C. albicans* is concerned.

There is a chapter on spores of saprophytic fungi as allergens. The book is most valuable as a general survey of the field. For finer points of mycology more comprehensive volumes should be consulted.

J. W. DOUGHERTY
New York, New York

BOOKS RECEIVED

THE DIABETIC'S COOKBOOK. By Clarice B. Strachan. Pp. 304. The Medical Arts Publishing Foundation, Houston, Texas, 1955, $6.50.

American Trudeau Society

Medical Section of the National Tuberculosis Association

Acceptable Standards in the Treatment of Tuberculosis

A Joint Statement of the Committees on Therapy and on Administrative Problems

This statement represents the joint and considered opinion of the American Trudeau Society Committees on Therapy and on Administrative Problems.

In recent years the discussion of hospital and home care of patients with tuberculosis has tended to cloud certain concepts which need re-emphasis.

Tuberculosis remains a chronic, infectious disease. It requires long and continuous treatment. The physician should be assured of the cooperation of the patient and family. This is necessary to be sure that the treatment and other recommendations made will be followed in compliance with good clinical and preventive medical practices and the regulations of the local Boards of Health.

Good medical practice requires the maximum facilities for clinical evaluation of a case, which, in most instances, can be done best in the hospital where appropriate treatment can be started. Preferably it should be continued until the patient's condition is satisfactorily stabilized. Now with more effective treatment methods, it may be reasonable to modify the period of hospitalization for certain selected cases. The treatment of tuberculosis remains difficult and the results in individual cases are frequently unpredictable. When home care is used, it must be carefully coordinated with hospital care, particularly during treatment of the active stages.

To secure the best results, certain standards must be met:

(*1*) *Medical care*. The patient should be under the continuous supervision of a well-trained physician or group of physicians, who thoroughly understand the care, management, and treatment of tuberculosis. As most cases will involve both hospital and home care, there must be maximum coordination of inpatient and outpatient services and careful cooperation with the private physician in the approach to this treatment.

(*2*) *Diagnosis*. Facilities must be available for the diagnosis and subsequent management of all patients. These would include readily available roentgenographic examinations with facilities for special examinations, such as stereoscopic films, planigraphy, fluoroscopy, and other needed measures. Provisions must be made for clinical laboratory examinations, biopsy, bronchoscopy, and other necessary tests so frequently required to make a correct diagnosis.

(*3*) *Isolation*. Tuberculosis remains a communicable disease and this fact needs to be kept continuously in mind. Facilities must be available for the isolation of the patient to protect the members of his family and the public. It must be remembered that sputum often does not become negative for *M. tuberculosis* during the first several months of treatment and too frequently fails to convert in advanced cases even under the best therapy.

(*4*) *Nursing*. Rest and nursing care must be assured for the patient. Provisions for rest must include both physical relaxation and psychological rest with all that this implies. Nursing care includes not only the physical aid given by the nurse to the patient, but also the assistance to the physician in the education of the patient concerning his disease and the necessity for treatment.

(*5*) *Nutrition.* A well-balanced diet is a necessity. Assistance in the selection and preparation of this diet will be needed, particularly in the early months of therapy.

(*6*) *Drug therapy.* Antimicrobial therapy must be available for long-term, continuous treatment in accordance with accepted regimens. The drug regimen should be determined on medical factors alone, and should not be influenced by the convenience of the patient, physician, or nurse. *Once started, antimicrobial treatment should be continued without interruption as long as medically indicated.*

(*7*) *Sputum examinations.* Laboratory facilities must be available to provide periodic examinations of sputum or gastric contents at regular intervals. This will vary from frequent examinations at the start of treatment to at least bi-monthly examinations after several months. Studies must include cultures at regular intervals, especially when smears are negative for *M. tuberculosis.* Cultures of positive sputum are necessary for diagnostic identification, viability, and drug-susceptibility tests. It is advisable to include the studies of the bacilli for drug susceptibility, and the results should be evaluated in conjunction with clinical data. Alterations in therapy should not be made hastily on the basis of these results alone. Other laboratory studies to detect early evidences of drug resistance should be made.

(*8*) *Surgery.* Surgical consultations should be held early and often, in view of the large number of patients who require surgery. There must be access without delay to hospitals well equipped for thoracic surgery.

(*9*) *Adjunct services.* All auxiliary services such as recreation, occupational therapy, education, medical social service, and rehabilitation should be available from the beginning of the treatment period.

(*10*) *Follow-up.* Long-term clinical, radiographic, and bacteriologic follow-up is essential after the patient returns to community life and should be available in order to detect a possible relapse.

(*11*) *Patient education.* It is essential for the patient to understand his disease for successful and permanent recovery. Patient education by the physician, assisted by the nurse and others, thus becomes of major importance in treatment.

The patient with tuberculosis needs all of the above-mentioned services during various periods of his disease and treatment. Neither home care nor hospital care that fails to provide these services can be considered adequate at this time. It has not yet been proved that even the less destructive forms of tuberculosis can be treated adequately without these aids, although many studies are now in progress.

Most patients would benefit and could be assured of better success if treatment were initiated and continued in a hospital for as long as indicated before being continued in the home. It seems obvious that these necessary facilities and services can best be provided in a hospital during the active stages of the disease. During subsequent phases, these services should be made available to the patient at home.

J. Burns Amberson
William R. Barclay
Paul T. Chapman
Raymond F. Corpe
Felix A. Hughes, Jr.
Carl Muschenheim
Robert L. Yeager
Morris C. Thomas, *Chairman, Committee on Therapy*

Herbert R. Edwards
Harold S. Hatch
Hilbert Mark
Richard Nauen
Cedric Northrop
Sumner S. Cohen, *Chairman, Committee on Administrative Problems*

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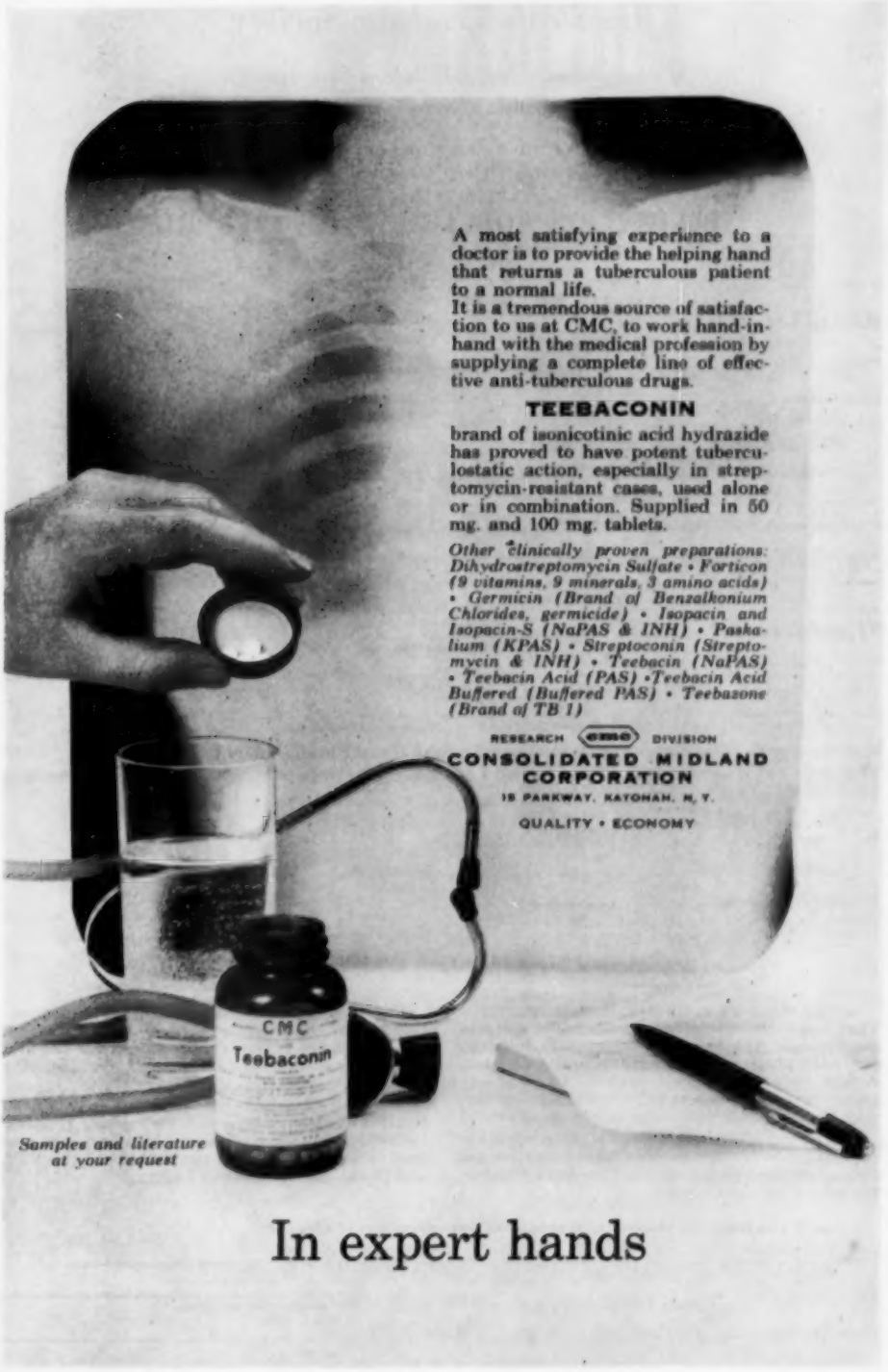
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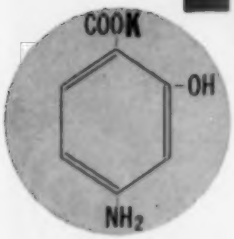

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1. *Molthan, L., Cohen, B. V., and Zarafonetis, C. J. D.: Clinical use of potassium para-aminosalicylate (KPAS). Am. Rev. of Tuberc. and Pulmon. Dis. 71:220 (Feb.) 1955.*

2. *Cohen, B. V., Molthan, L., and Zarafonetis, C. J. D.: Clinical Studies of Various Forms of PAS (with special reference to plasma concentrations). Read before the American Trudeau Society, Annual Meeting, Milwaukee, Wisconsin, May 23-27, 1955.*

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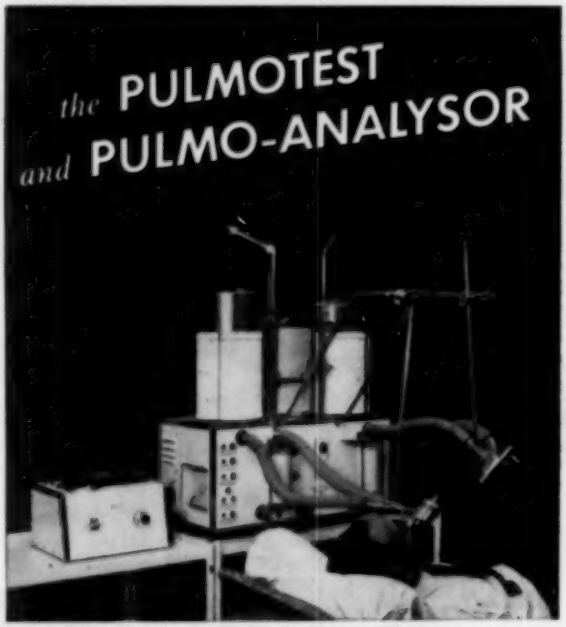

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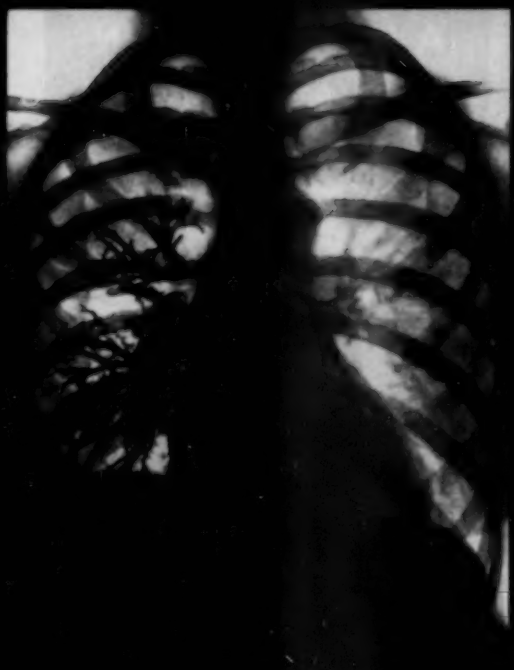

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1. Burrascano, J. J.: Sea View Hosp. Bull. *15*:149 (July) 1955.

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1. Wolfson, W. Q.: Mississippi Valley M. J. 77: 66, 1955.

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1. Recommended Dietary Allowances, Washington, D. C., National Academy of Sciences—National Research Council, Publication 302, 1953.
2. Co Tui: Review: The Fundamentals of Clinical Proteinology, J. Clin. Nutrition *1*:232 (Mar.-Apr.) 1953.
3. Keys, A.; Brozek, J.; Henschel, A.; Mickelsen, O., and Taylor, H. L.: The Biology of Human Starvation, Minneapolis, Univ. of Minnesota Press, 1950.
4. Burger, G. C. E.; Drummond, J. C., and Sandstead, H. R.: Malnutrition and Starvation in Western Netherlands, The Hague General State Printing Office, 1948, Part II, p. 91.
5. Co Tui; Kuo, N.H.; Chuachiaco, M., and Mulholland, J.H.: The Protein Depletion (Hypoproteinia) Syndrome and Its Response to Hyper-Proteinization, Anesth. & Analg. *28*:1 (Jan.-Feb.) 1949.

The nutritional statements made in this advertisement have been reviewed and found consistent with current medical opinion by the Council on Foods and Nutrition of the American Medical Association.

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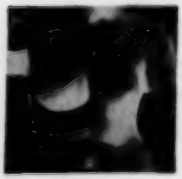

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